Development of gene therapy strategies in rodent models of retinal degeneration

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A thesis submitted for the degree of
Doctor of Philosophy, University of London

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2006

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Declaration:

I, Prateek Buch, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been acknowledged in the thesis.

Signed:

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Abstract

Inherited retinal dystrophies constitute one of the most prevalent causes of bilateral visual loss, affecting as many as 1 in 3000 people. Genetic therapies have been developed following the identification of disease-causing mutations, using an understanding of how these mutations cause retinal degeneration and improved viral vectors designed to give safe and efficient gene delivery. Hence a broad range of retinal disorders may be treatable with gene therapy, although many aspects of ocular gene therapy warrant further investigation.

The studies presented here aim to improve gene therapy in two well-characterised models of retinitis pigmentosa, the *Prph2*$^{Rd2/Rd2}$ mouse and the RCS rat. Gene replacement therapy can be effective in these and other models, mediating both structural and functional improvement. However, in many cases long-term rescue from degeneration is not achieved, as evidenced by a continuing loss of photoreceptor cells. These studies aim to improve adeno-associated virus (AAV)-mediated gene delivery to the murine retina by using novel promoters to investigate the consequences of gene replacement therapy. Additionally an alternative viral vector (AAV2/5) will be used to enhance gene replacement, which gives faster and stronger transgene expression than the AAV2/2 used in previous studies.

Cell death can also be prevented by administration of vectors expressing neurotrophic factors, but no study to date has identified such a factor that enhances gene replacement therapy; indeed, AAV-mediated *CNTF* expression has serious deleterious effects on retinal function. Hence here we have investigated these deleterious effects, and determined whether CNTF can be used to prevent photoreceptor death without the reduction in retinal function seen previously. In addition, the ability of AAV-mediated *GDNF* expression to enhance gene replacement therapy in both the *Prph2*$^{Rd2/Rd2}$ mouse and the RCS rat has been evaluated in detail. These studies show for the first time that neurotrophic factor delivery, when used in combination with gene replacement therapy, can enhance structural and functional improvement in models of retinitis pigmentosa.

Contents

Abbreviation List

Below is a list of technical terms and common abbreviations used in this thesis. As far as is possible the terms are explained in full when first introduced, and subsequently used in abbreviated form.

AAV	Adeno-associated virus
Ad	Adenovirus
AMD	Age-related macular degeneration
BDNF	Brain-derived neurotrophic factor
cGMP	cyclic guanosine monophosphate
CMV	Cytomegalovirus
CNTF	Ciliary neurotrophic factor
DMEM	Dulbecco's modified Eagle medium
ERG	Electroretinogram
FGF	Fibroblast growth factor
GDNF	Glial cell line-derived neurotrophic factor
GFP	Green fluorescent protein
HIV	Human immunodeficiency virus
Lenti	Lentiviral vector
MOPS	Mouse opsin promoter fragment
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEDF	Pigment epithelium-derived factor
Prph2	Peripherin 2
Rho	Rhodopsin promoter (bovine)
RPE	Retinal pigment epithelium

SFFV	Spleen focus-forming virus
SIN	Self-inactivating
WPRE	Woodchuck post-transcriptional regulatory element

Tables and Figures

Acknowledgments

I would firstly like to thank Professor Robin R. Ali for his support, guidance and patience in supervising my work; it has been a privilege to be part of such a successful team.

I have received a lot of technical assistance as part of the studies presented here. In particular I would like to thank Dr. Alexander Smith for invaluable guidance, advice and laboratory training. In addition I must thank Yanaí Durán and Marion Tschernutter for carrying out much of the histology in Chapters 4 and 5, YD for assistance with electrophysiology, Drs. Kam Balaggan and James Bainbridge for performing sub-retinal surgery and Dr. Angus Macneil for sub-cloning one of the plasmids described in Chapter 5. AAV2/5 production was developed with the help of Dr. Fabienne Rolling (INSERM, Nantes, France) and Dr. Amit Nathwani (University College London), the reporter gene data using AAV2/5 is presented courtesy of Dr. Mythili Rajah, whilst the integrase-defiecient lentiviral vectors were developed by Dr. Rafael Yañez-Muñoz (University College London). Many thanks also to Dr. Rachael Pearson for help with confocal microscopy and immunohistochemistry, and to Dr. Robert MacLaren for assistance with much of the work in Chapter 5.

I have enjoyed so much support and love from my parents, great friendship and encouragement from my brother and such love and care from my wife that without them the work here would not have been possible. "Karmanye Vadhikaraste, Ma Phaleshu Kadachana..."

To everyone mentioned here, and the countless others who aren't, I offer my sincere gratitude.

1. Introduction

1.1 The eye – structure, function and genetic disease

Vital to the ability of higher organisms to sense, evaluate and interact with their environment, the eye is often referred to as the one of the most complex organ systems in biology. The primary function of the human eye is to respond to visual stimuli by transducing a response to light into neuronal signals that are interpreted by the brain. The eye carries out many disparate cellular and molecular processes to support the sensing of external stimuli; a tightly regulated microenvironment is maintained within the eye with regards to the differentiated status of its various cell types, vasculogenesis and modulation of the local immune system. This complexity of function arises from the variety of developmental origins of the cell types within the eye, and leads to a high degree of sensitivity to genetic and environmental factors. Slight changes in the finely balanced pattern of gene expression can lead to disease, as can subtle environmental insults and infections. The neurosensory retina, which responds to light entering the eye by initiating a neuronal signalling cascade, is the seat of many inherited genetic diseases, many of which result in blindness in one or both eyes and have been extensively researched in both clinical and laboratory settings. However, despite increased knowledge of aetiology and pathology of disease, many ocular conditions, especially retinal disorders, lack effective treatments.

1.2 Structure of the eye

The mammalian eye consists of concentric layers of tissue derived from two of the three embryological layers, the ectoderm and the mesoderm. An outer, fibrous casing, the sclera, forms a tough barrier that protects the eye; this layer originates in the mesoderm. Contiguous with the sclera, and also arising from the mesoderm, the anterior part of the eye's outermost layer is the cornea (for schematic diagram see **Fig. 1.1**). At the anterior opening of the eye, posterior to the cornea, lies the lens, which alters the refractive index of incident light by changing shape; this ectoderm-derived tissue enables images from varying distances to be focused onto the light-sensitive

neuroretina. Encased inside the sclera is a vascular layer of tissues, amongst which the iris and choroid are found. Adjacent to this layer lies the neuronal tissue known as the neurosensory retina. This photosensitive tissue is where the capture, amplification and transduction of light signals to the visual cortex takes place [1], and the retina has evolved to carry out these primary functions of the eye as discussed below.

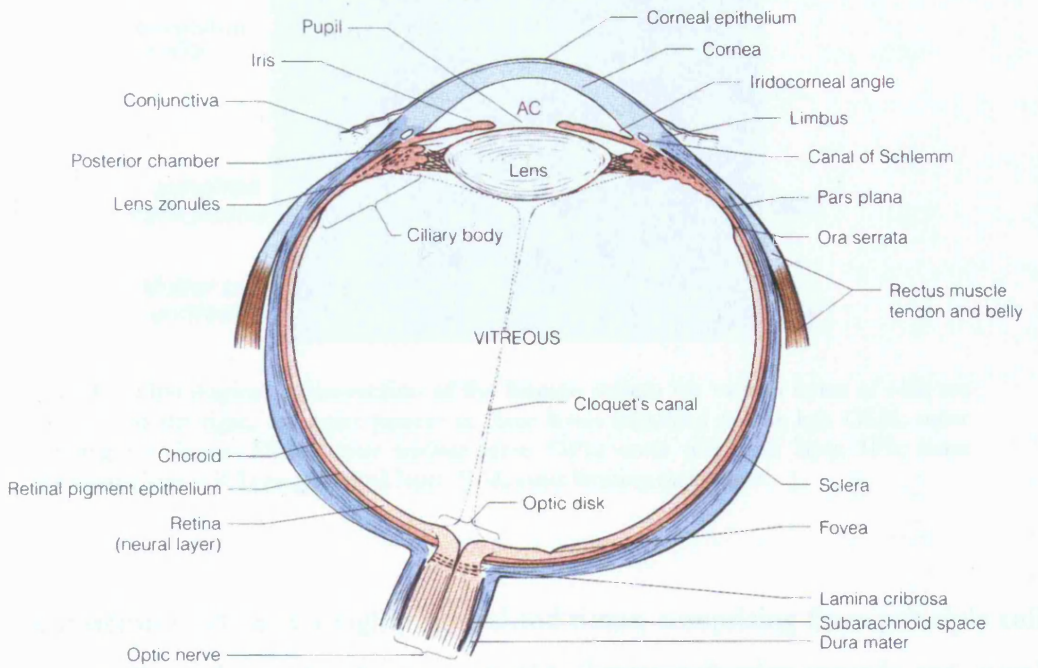


Fig. 1.1: Structure of the eye; schematic diagram showing features of the human eye. [1]

1.3 The retina – structure and function

Consisting of several distinct layers of cells, the retina carries out the various functions required to achieve phototransduction – the process by which light is received and converted into neuronal impulses. This neural layer of cells is generally viewed as two distinct tissue compartments: the inner, neurosensory retina, and the outer, supporting layer of cells, the retinal pigment epithelium (RPE).

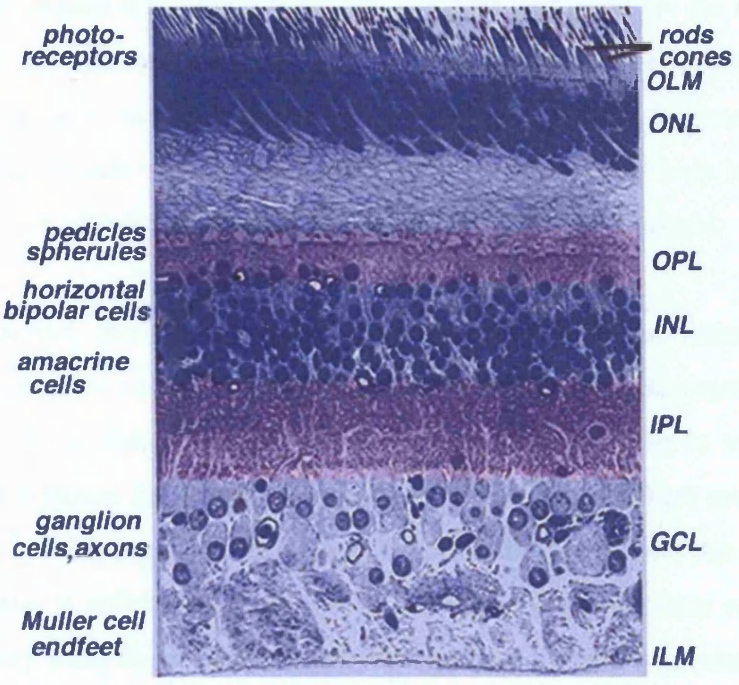


Fig. 1.2: Histological cross-section of the human retina; the various layers of cells are labelled on the right, cell types present in these layers identified on the left. OLM; outer limiting membrane. ONL; outer nuclear layer. OPL; outer plexiform layer. IPL; inner plexiform layer. GCL; ganglion cell layer. ILM; inner limiting membrane.

The neurosensory retina is a highly specialised tissue, comprising three principle cell types that perform the central functions in the phototransduction cascade and visual pathway: photoreceptor cells, bipolar cells and ganglion cells. The cells that respond to light entering the eye are the photoreceptor cells, of which the mammalian eye contains two different types: rods and cones. Rod- and cone-cell nuclei and cell bodies are found in the outer nuclear layer of the eye as seen in histological cross-section (**Fig. 1.2**), and rods dominate the photoreceptor cell population in the mammalian eye; there are around eighteen times as many rods as cones in an adult human eye. Rod and cone photoreceptors differ in the visual pigment they contain, and as a result they respond to different stimuli; rods contain the pigment rhodopsin whilst cones house different cone opsins depending on which sub-type of cone cell they belong to. Rods are sensitive to conditions of dim light and are responsible for scotopic (night) and peripheral vision, whereas cones respond to bright light of varying wavelengths allowing colour vision. In humans cone cells are also responsible for central visual acuity due to their spatial distribution (see below). Humans have

three sub-types of cones, which respond to different wavelengths of light. Those that are maximally sensitive to long wavelengths of light (red light) are the L-cones, cones responsive to medium wavelengths of light (green light) are the M-cones, and cones that respond to short wavelengths of light (blue light) are the S-cones. Rodents and most other mammalian species are di-chromatic, meaning they only have cone cells that respond to medium- and short-wavelength light (M- and S-cones).

In the human eye, there is a central, pigmented portion of the retina, the macula, which is enriched for cone cells. Approximately 5 mm in diameter, and located 3 mm lateral to the optic disk (**Fig. 1.1**), the macula contributes most to visual acuity in humans, as it contains the greatest concentration of cone cells; there are 150,000 cone cells per mm^2 in this region of the human eye. Within the macula, the central portion of retinal tissue is referred to as the *fovea centralis*. Here the inner retinal cells are displaced away from the underlying photoreceptors, allowing a greater proportion of incident light to be absorbed by the cells in this region (**Fig. 1.3**); this also contributes to the increase in acuity in this central retinal area. Rodents and other mammals (other than non-human primates) lack an anatomically defined macula, with cones distributed more evenly throughout the retina; this has implications for the study of retinal disease in animal models, as discussed below.

Photoreceptor cells have distinct cellular structures, comprising inner and outer segments, as well as a synaptic region and a cell body that contains the nucleus (**Fig. 1.4**). The inner segments are densely packed with mitochondria, and provide a metabolic centre from which the energy required for the process of phototransduction is derived. A connecting cilium leads from inner to outer segment, which is intimately involved in the regulated transport of molecules into and out of the outer segment. In both rods and cones the outer segment is where their respective visual pigments are localised, which absorb light in a manner discussed below (**Chapter 1.4**). The outer segments in cone cells are made up of invaginations of the plasma membrane, known as lamellae. In rods these invaginations undergo further folding to form stacks of membranous discs discrete from the plasma membrane [2]. Inserted into cone lamellae and rod discs are the structural proteins peripherin-2 and rom-1 (see **Chapter 1.6),** both of which are required for the formation and maintenance of outer segments.

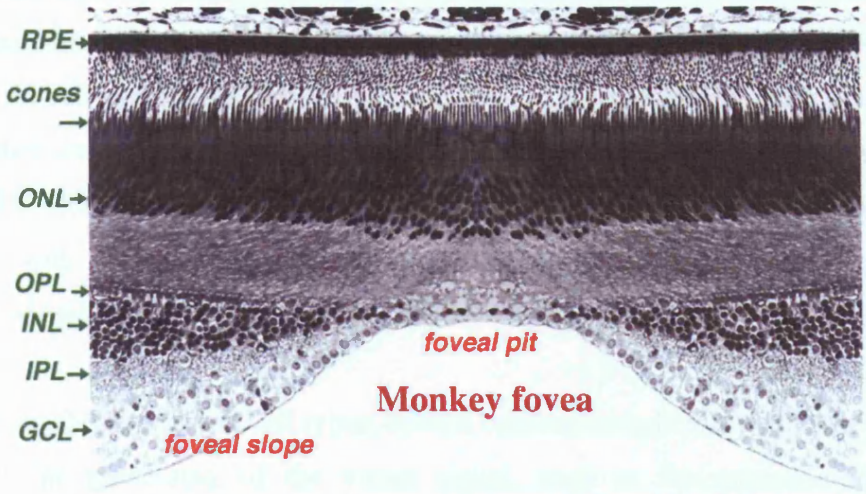


Fig. 1.3: Retinal cross-section of primate fovea; inner retinal cells are displaced from the foveal pit, allowing more light to reach cone photoreceptors located in the *fovea centralis*.

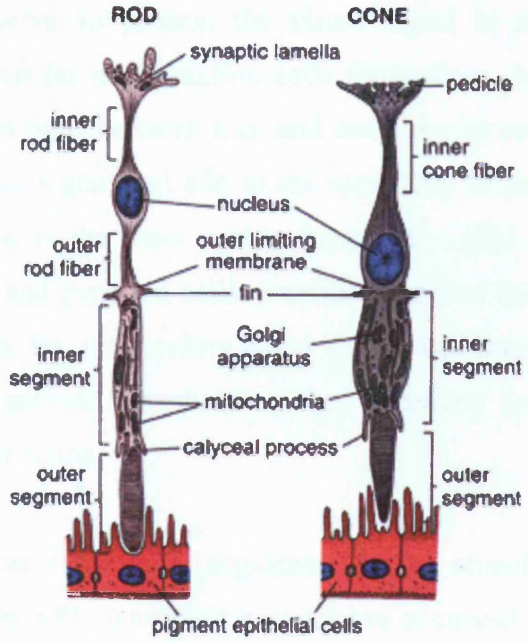


Fig. 1.4: Rod and Cone photoreceptor cells; schematic diagram showing properties of both types of photoreceptor cell. Taken from http://anatomy.iupui.edu/

Using connections at their innermost aspect, photoreceptor cells synapse with bipolar cells in the inner nuclear layer (**Fig. 1.2**), each of which receive stimuli from several photoreceptors. These bipolar cells in turn project axons into the inner plexiform layer of cells in the retina, and synapse with dendritic processes of ganglion cells (whose cell bodies lie in the ganglion cell layer, anterior to the inner plexiform layer – see **Fig. 1.2**). The axons of the ganglion cells form the fibres of the optic nerve, and synapse with cells lateral geniculate nucleus; from here impulses are sent to the primary visual cortex.

Aside from these principal cell types, several other neuronal cells within the retina are required for processing of the visual signal, such as the amacrine association neurones. There are at least 20 morphologically distinct sub-classes of amacrine cell, which can be further classified according to the neurotransmitter they secrete (generally Gamma-Aminobutyric acid (GABA) or dopamine). Amacrine cells lack anatomically defined axons, but have dendrites that synapse with rod bipolars, from which they receive a signal in response to light which they then transmit to retinal ganglion cells (RGC); these represent horizontal connections between bipolar and ganglion cells, and serve to process the visual signal in addition to the direct connection between bipolar and ganglion cells themselves. As amacrine cells also make horizontal connections between rod- and cone-bipolar cells, they contribute to processing of the visual signal and add to the sensitivity to movement and contrast [3]. Müller cells, found in the inner nuclear layer, are a glial cell type that support both bipolar neurones and ganglion cells, providing survival factors and nutrients for these inner retinal cells. By surrounding blood vessels and insulating neuronal axons and cell bodies, they provide essential functions necessary for the maintenance of both the inner and outer retina.

Posterior to the photoreceptor outer segments lies the retinal pigment epithelium (RPE, see **Fig. 1.2**). The RPE comprises a monolayer of cuboid cells, the basal aspect of which lies on the connective tissue layer known as Bruch's membrane. The RPE plays a vital role in the eye, providing much of the trophic support required to maintain the photosensitive retinal cells; components of the inter-photoreceptor matrix and various growth factors are produced by the RPE, which are essential for the development and support of rods and cones. In addition, RPE cells phagocytose

photoreceptor outer segments, allowing them to be turned over and re-synthesised. The RPE is also involved in the visual cycle, essential for the uptake and turnover of by-products of the phototransduction cascade, as discussed in more detail later (**Chapter 1.4**). The RPE is thus crucial for effective maintenance of the neuronal component of the retina, and hence some diseases with retinal phenotypes in fact arise from primary defects in RPE function (**Chapter 1.6**).

Despite the lack of an anatomically defined macula in rats and mice, and the different spatial distribution and density of cone cells, the murine retina remains a useful model system in which human retinal dystrophies may be studied. Many of the genes that cause hereditary retinal disorders in humans either have naturally occurring counterparts in rodents, or have been modelled by using transgenic, knock-out or selective gene ablation technologies [4]. By studying the pathophysiology of such models, the mechanism of disease can be elucidated in many cases, and valuable insights into human disease have been obtained through investigations in animals. However, the differences between human and animal retinal biology must always be considered when applying conclusions from animal experiments to clinical contexts; the relevance of rodent disease mechanisms and responses to treatment is always open to question.

1.4 Phototransduction and the visual cycle

Neuronal signalling in response to an external stimulus is generally associated with a depolarisation of the actively maintained voltage across the cell membrane. This in turn results in secretion of a neurotransmitter that is detected by post-synaptic cells. In the case of photoreceptors, however, in the resting (un-stimulated) state the plasma membrane is depolarised, and the neurotransmitter glutamate is released into the synaptic space where it is bound by receptors on post-synaptic bipolar cells. This is an important consideration when discussing the phototransduction cascade; the 'ground state' for rod and cone cells is one of a depolarised, actively secreting cell, which upon stimulation reverts to a hyperpolarised state, meaning that it is the *reduction* in neurotransmitter release that gives rise to subsequent signalling involved

in perception of light by the brain. The depolarised 'ground state' and the hyperpolarised 'excited' state are shown schematically in **Fig. 1.5.** Once light enters the eye, it reaches the photoreceptor cells where it is absorbed by the visual pigment housed in membranous discs of the outer segments. In the case of rod cells, this pigment is rhodopsin; this is a G-protein coupled receptor (GPCR) that consists of a seven-pass transmembrane protein, rod opsin. As shown in **Fig. 1.6**, the seventh intradiscal domain is associated with the Vitamin A analogue 11-*cis* retinal; this is the chromophore element of both rhodopsin and cone opsin, and when photons of light are absorbed by 11-*cis* retinal, it isomerises to all-*trans* retinol.

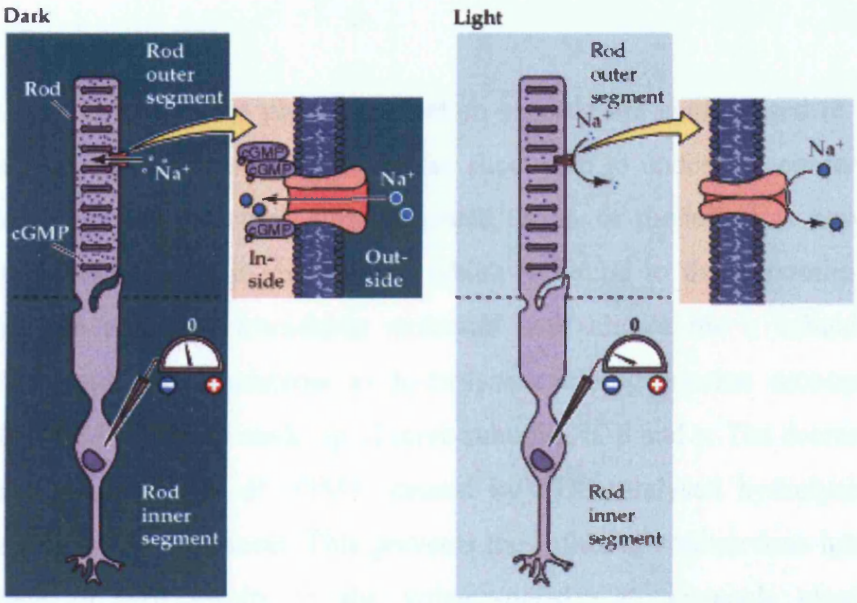


Fig. 1.5: Ion transport and membrane potential in photoreceptors in dark and light states; In the dark, cGMP-gated sodium channels allow an influx of Na^+ ions into the photoreceptor, maintaining a depolarised membrane potential (Dark, left panel). This depolarisation means that voltage-gated calcium channels (not shown) remain open in the dark. Upon light excitation, reduced levels of cGMP lead to closure of the Na^+ channels, and subsequent closure of voltage-gated Ca^{2+} channels, hyperpolarising the photoreceptor (Light, right panel).

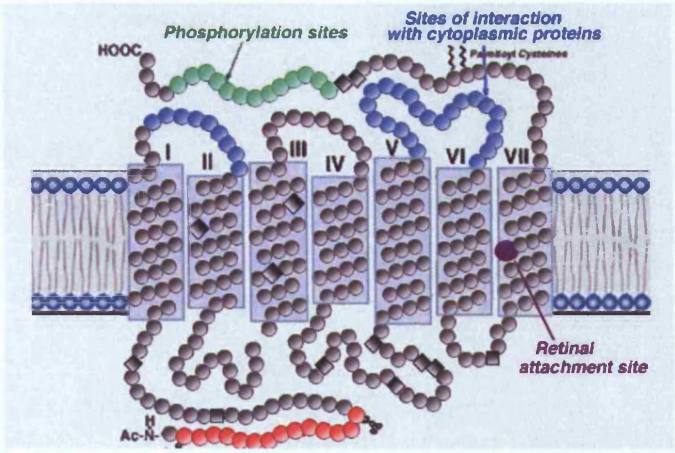


Fig. 1.6: Tertiary structure of rhodopsin; Highlighted are 11-*cis* retinal binding site (purple) phosphorylation sites used by rhodopsin kinase (green), glycosylation sites (red), and arrestin interaction sites (blue). From http://webvision.med.utah.edu/photo1.html

The subsequent steps in the phototransduction cascade are summarised in **Fig. 1.7**. The isomerisation to all-*trans* retinol causes rhodopsin to undergo a conformational change in its tertiary structure; this 'activated' form of rhodopsin is now able to activate the small G-protein, transducin, which is bound to the C-terminal end of rhodopsin. The activated transducin molecule then allows the γ subunit of the phosphodiesterase (PDE) enzyme to hydrolyse cyclic guanosine monophosphate (cGMP) to 5'GMP. PDE is made up of three subunits, α, β and γ. The decrease in the intracellular concentration of cGMP, caused by PDE-catalysed hydrolysis, closes cGMP-dependent Na^+ channels. This prevents the influx of sodium ions into the rod cells, which in turn results in the voltage-gated Ca^{2+} channels closing. The photoreceptor cell is now hyperpolarised; as a result, the neurotransmitter glutamate, which is continuously released in unstimulated conditions, is prevented from being released. This drop in glutamate release by the photoreceptor cells upon light stimulation is sensed by the post-synaptic bipolar cells, which respond by either depolarising or hyperpolarising their own synaptic membranes, depending on whether they are ON- or OFF-bipolar cells. These bipolar cells then signal to retinal ganglion cells, whose axons project from the posterior pole of the eye at the optic disk to form the optic nerve; these axons extend along the optic tract and synapse with neurones in the lateral geniculate nucleus, from where impulses are sent to the primary visual cortex for interpretation of the image sensed initially by the retina.

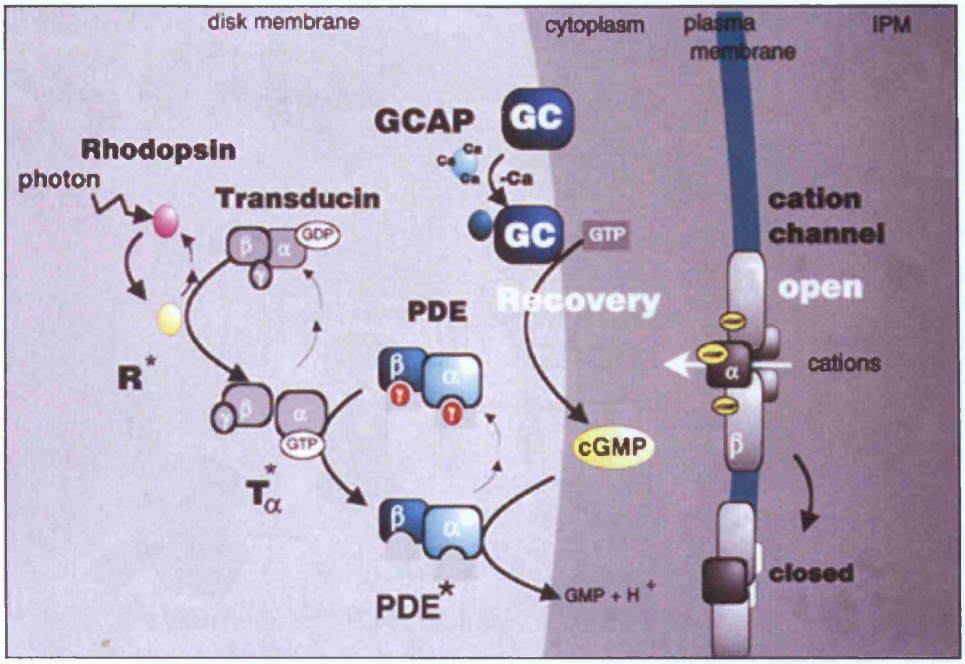


Fig. 1.7: Cartoon of major steps in phototransduction; Light induces structural change in rhodopsin; GTP binds to PDE; cGMP converted to 5'GMP; cGMP-gated Ca^{2+} channels close; glutamate release prevented.
From http://depts.washington.edu/ophthweb/KPgraphpic.html

Termination of the phototransduction cascade, along with the recycling of the many components involved, is required to prevent over-stimulation of the photoreceptors and also to ensure visual information can be continuously processed in conditions of prolonged stimulus. The all-*trans* retinol moiety of the rod cell visual pigment, synthesised by the isomerisation of the 'resting' 11-*cis* retinal, cannot bind the opsin apo-protein. Instead, once the activated rhodopsin molecule has initiated transducin activation, the all-*trans* retinol dissociates from the transmembrane protein and is transported into the adjacent RPE cells by the transport protein ABCA4, formerly known as ABCR. In the RPE cells, a series of NADPH-dependent reactions drive the conversion of the all-*trans* form back to 11-*cis* retinal; this involves the microsomal protein RPE65, and the enzymes lecithin:retinol acetyl transferase (LRAT) and 11-*cis* retinol dehydrogenase (11-*cis* RDH, see **Fig. 1.8**). The 11-*cis* retinal is then recovered to the photoreceptor outer segment; this step involves the inter-photoreceptor retinol binding protein (IRBP), which binds the retinal and shuttles it into the photoreceptor. Here it associates with rhodopsin molecules anchored to the outer segment discs.

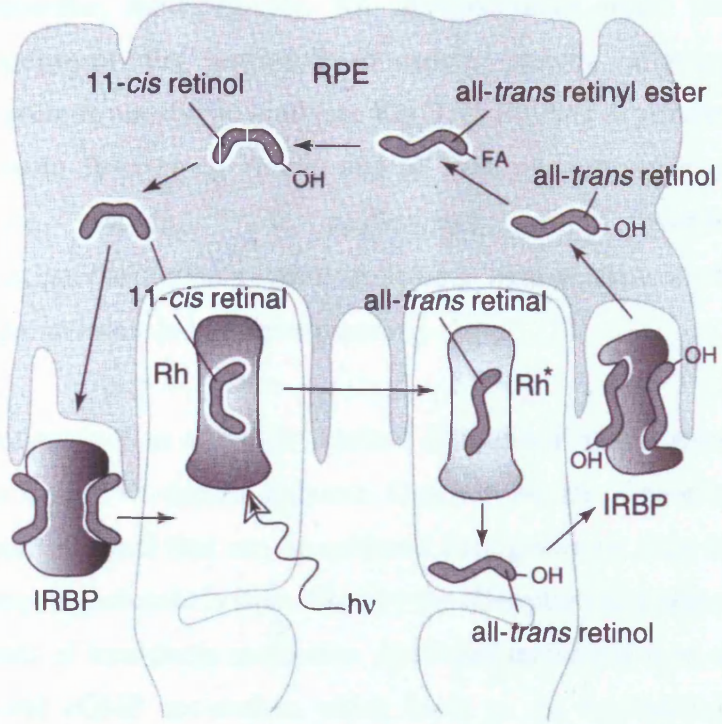


Fig. 1.8: Regeneration of visual pigment in retinal pigment epithelium cell; once light (hv) is absorbed by rhodopsin (Rh), the chromophore 11-*cis* retinal isomerises to all-*trans* retinol. Following uptake of this molecule into the RPE, enymatic reactions lead to the regeneration of 11-*cis* moiety via an ester intermediate. This molecule is then transported back into the photoreceptor by IRBP. Diagram adapted from one available at http://biophysics.bumc.bu.edu/faculty/cornwall/index.html

In order to halt the phototransduction cascade upon light stimulation, and to return the photoreceptor cells to their 'ground state' of depolarisation, the cGMP-dependent Na^+/Ca^+ channels need to be closed. To achieve this, retinal guanylate cyclase (RetGC, encoded by the gene *GUCY2D*) is activated when cGMP levels fall; this activation is mediated by the calcium-binding proteins known as the Guanylate Cyclase Activating Proteins (GCAP), which are part of the calmodulin-like superfamily of calcium binding proteins [5]. RetGC converts 5'GMP back to the cyclic form, allowing cGMP-gated channels to re-open and stop the visual pathway from being activated. In addition to the action of RetGC, photorecovery is mediated by de-activation of rhodopsin at the outer segment disc membrane. The rhodopsin kinase (RK) enzyme, part of the G-protein receptor kinase (GRK) family, is a serine/threonine protein kinase whose phosphorylation activity is limited to the active

form of its substrate, rhodopsin [6]. RK phosphorylates serine residues in the cytoplasmic loops of the activated rhodopsin protein, allowing a second photorecovery protein, arrestin, to bind (see **Fig. 1.7**). Binding of arrestin to rhodopsin prevents transducin from being bound, and so halts downstream signalling in the phototransduction cascade. These mechanisms prevent overstimulation of photoreceptor cells; the period of time following light-excitation where the cells return to their ground state is known as recovery.

Thus the phototransduction signalling cascade is initiated by the absorption of one photon of light by one rhodopsin molecule. One key feature of phototransduction is the amplification of signal that can be achieved during various steps in the cascade. Once one rhodopsin molecule is isomerised by the absorption of a photon, it is able to activate hundreds of transducin molecules. Activated transducin then allows PDE to hydrolyse several cGMP molecules, which leads to the hyperpolarisation of the photoreceptor of around 1 mV. Thus only a few photons of light need be absorbed for a rod cell to become hyperpolarised.

This is how light sensitive cells within the retina are able to convert the energy from photons of light into biochemical signals, resulting in neuronal excitation through to the visual cortex. Defects in genes encoding protein involved in many steps of the mechanism described above can lead to diseases that vary in severity and clinical symptoms. The most common outcome of these mutations is uni- or bi-lateral loss of vision. Many of these defects can be inherited, and it is on a family of these heritable retinal diseases that the current studies focus.

1.5 Inherited retinal degeneration

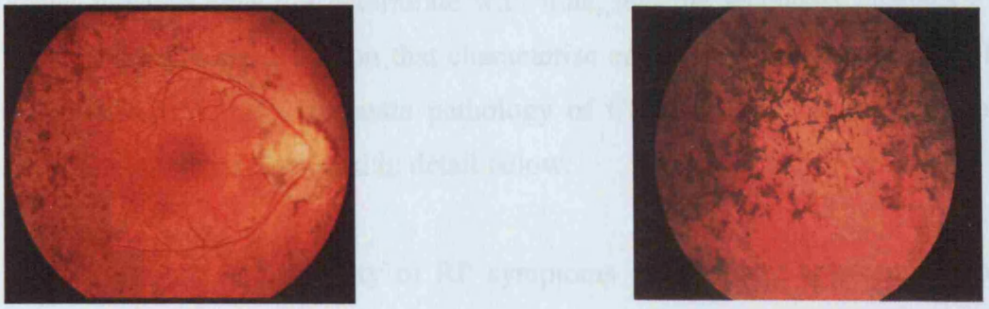
Inherited retinal disorders, including retinitis pigmentosa (RP), affect as many as 1/3000 people worldwide [7], and currently there are no effective treatments available. These retinal dystrophies can be classified according to their clinical phenotype, and now according to their underlying molecular defects. Clinically the

disorders present as either stationary or as progressive conditions; the former being caused by dysfunctional photoreceptors, the latter by the death of photoreceptors due to their underlying mutations. The different mutations causing these conditions are discussed below. These retinal dystrophies are further sub-classified according to the exact cell type affected. Rod cell dystrophies are caused primarily by the disruption of function and/or death of rod cells, whilst the cone population remains largely intact. Cone dystrophies affect only cones, with visual acuity and colour vision impaired but night vision remaining normal. Some inherited retinopathies involve loss of both cone and rod cells, and are called cone-rod or rod-cone dystrophies, depending upon which photoreceptor subtype is primarily affected. Of note is that many of these dystrophies involve both types of photoreceptors at advanced stages of disease, culminating in the loss of central visual acuity that is the primary cause of loss of quality of life in patients.

Characterised by loss of scotopic (night) vision, followed by peripheral visual loss and finally macular degeneration, retinitis pigmentosa is a term that encompasses a range of molecular defects affecting the structure, function and maintenance of (primarily) rod photoreceptors. Inherited as an autosomal recessive (arRP), autosomal dominant (adRP) or as an X-linked trait, a feature of RP is the extraordinary genetic heterogeneity seen in disease. Hundreds of different mutations in 36 photoreceptor-specific and RPE-specific genes (and 11 genetic loci) have been shown to segregate with incidence of disease in human pedigrees [8]; conversely, families in which the same mutation causes visual loss can show a range of clinical presentations, with variation in time of onset, penetrance and severity of visual loss.

The clinical signs of disease are varied, and particular symptoms of retinitis pigmentosa are associated with the mode of inheritance and sub-type of RP concerned. Fundus examinations of patients do, however, reveal clear common signs of disease, and the clinical course of RP can hence be followed non-invasively. Beginning with the narrowing of retinal arterioles, disease progression is confirmed with the appearance of 'bone spicules' (caused by proliferating RPE cells migrating into the retinal space vacated by dead photoreceptor cells, **Fig. 1.9A**, ref. [9]). The presence of dense hyper-pigmented regions throughout the fundus is characteristic of more advanced RP (**Fig. 1.9B**).

Fig. 1.9: Fundus photographs from RP patients:
(a) Bone spicules in early RP (b) Dense pigmentation in advanced RP



Measuring the electrophysiological response of the retina to varying intensities of light provides an additional measure of retinal dystrophy. RP patients present with markedly reduced rod ERG amplitudes, as well as increased latency in cone b-waves in later stages of disease (**Fig. 1.10**) [10,11]. Poor sensitivity to low levels of light (often manifesting as night blindness) and narrowing of visual fields are often the first symptoms seen in patients with RP, whereas central visual acuity remains largely intact during the earlier stages of disease. This reflects the late onset of cone cell atrophy in RP pathology.

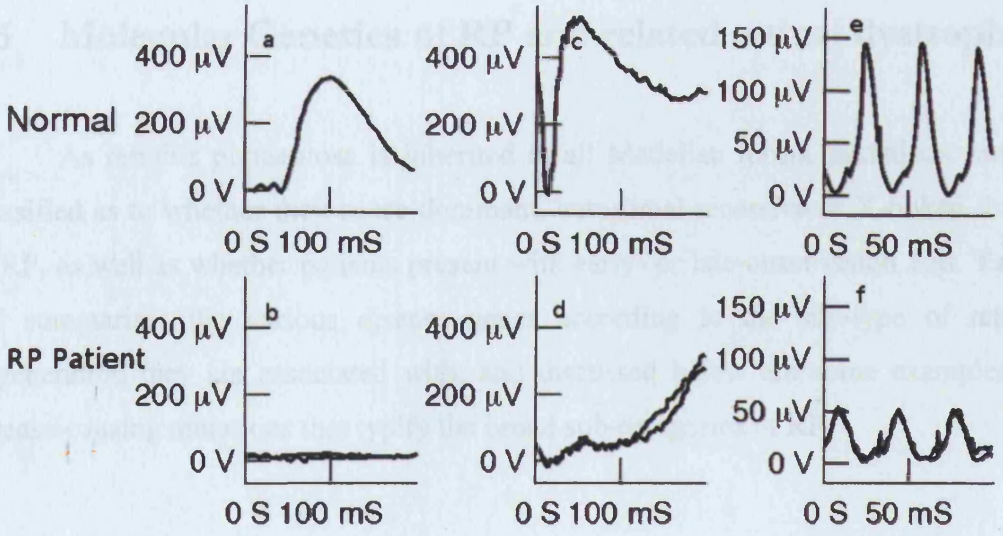


Fig. 1.10: Electroretinogram (ERG) recordings taken from normal individual and from RP patient; Scotopic (a and b), photopic (c, d) and 30Hz flicker (e, f) responses from an individual with normal vision (a, c, e) and from and RP patient (b, d, f). Patient shows extinguished scotopic b-wave, greatly reduced photopic a- and b-wave, and greatly reduced flicker response.

The impaired scotopic vision seen in RP is also present in the various forms of congenital stationary night blindness (CSNB). However, CSNB patients' reduced visual function does not deteriorate with time, and the secondary changes to retinal vasculature and pigmentation that characterise end-stage RP do not occur. This may reflect the potentially disparate pathology of CSNB-causing mutations versus RP-causing mutations, discussed in detail below.

The appearance and severity of RP symptoms vary greatly between patients. This variation reflects the heterogeneity in the genetics of RP, with different causative mutations giving rise to different phenotypes. For instance, X-linked RP is sub-categorised into RP2 and RP3, diseases linked to two distinct genetic loci (see **Chapter 1.6**). Both forms present with nyctalopia (loss of night vision) as early as 10 years of age [12], followed by loss of visual acuity in the thirties. Contrary to this, autosomal recessive forms of RP present with nyctalopia, poor night vision and peripheral visual loss from a later age, on average in the third decade of life; patients then go on to lose visual acuity, but not until their fifties. This clinical variation is compounded by syndromic RP-related conditions, where RP-like clinical presentation is coupled with non-related symptoms such as deafness in Usher's syndrome.

1.6 Molecular Genetics of RP and related retinal dystrophies

As retinitis pigmentosa is inherited in all Medelian forms, mutations can be classified as to whether they cause dominant, autosomal recessive or X-linked forms of RP, as well as whether patients present with early- or late-onset vision loss. **Table 1.1** summarises the various disease genes according to the sub-type of retinal degeneration they are associated with, and discussed below are some examples of disease-causing mutations that typify the broad sub-categories of RP.

Disease Category	Mapped and Identified Genes
Cone or cone-rod dystrophy, autosomal dominant	**AIPL1, CRX, GUCA1A, GUCY2D, RIMS1, SEMA4A, UNC119**
Cone or cone-rod dystrophy, autosomal recessive	**ABCA4, CNGB3, RDH5**
Cone or cone-rod dystrophy, X-linked	**RPGR**
Leber congenital amaurosis, autosomal dominant	**CRX, IMPDH1**
Leber congenital amaurosis, autosomal recessive	**AIPL1, CRB1, CRX, GUCY2D, LRAT, RDH12, RPE65, RPGRIP1, TULP1**
Retinitis pigmentosa, autosomal dominant	**CA4, CRX, FSCN2, GUCA1B, IMPDH1, NRL, PRPF3, PRPF8, PRPF31, RDS, RHO, ROM1, RP1, RP9, SEMA4A**
Retinitis pigmentosa, autosomal recessive	**ABCA4, CERKL, CNGA1, CNGB1, CRB1, LRAT, MERTK, NR2E3, NRL, PDE6A, PDE6B, RGR, RHO, RLBP1, RP1, RPE65, SAG, TULP1, USH2A**
Retinitis pigmentosa, X-linked	**RP2, RPGR**

Table 1.1: Genes in which mutations are known to cause retinal dystrophies; categorised here according to the disease they are associated with.
Taken from **http://www.sph.uth.tmc.edu/Retnet/sum-dis.htm#B-diseases**

1.6.1 Autosomal dominant RP

Mutations in the *RHODOPSIN* gene were first shown to cause autosomal dominant RP (adRP) in 1990, when Dryja *et al.* published a Pro-His substitution mutation in individuals with autosomal dominant RP [13]. Subsequent studies have revealed a large number of mutations in the *RHODOPSIN* gene, which in fact account for around 25% of RP cases in total [14]; over 100 different mutations have now been described, with significant phenotypic variation between them [15]. Mutations in *RHODOPSIN* are classified according to the biochemical properties of the mutant protein. Some mutant forms of rhodopsin appear to be gain-of-function mutants that fold correctly and localise to the photoreceptor outer segment (Class I mutants), and these versions are able to bind 11-*cis* retinal *in vitro* [16]. Class II mutants are generally misfolded and retained at the endoplasmic reticulum, leading to aggregation of the misfolded protein and the initiation of its ubiquitin-driven degradation. Indeed, this mislocalised and/or misfolded Rhodopsin protein may provide a distinct trigger for apoptosis in

certain forms of RP (see **Chapter 1.9**). As both Class I and Class II mutations in *RHODOPSIN* lead to a severe alteration in photoreceptor function and /or structure, they lead to progressive degeneration seen in retinitis pigmentosa, as opposed to stationary night blindness (see **Chapter 1.9** for further discussion).

Mutations in genes encoding photoreceptor-specific structural proteins also cause RP. Indeed, as rhodopsin is an integral membrane protein within rod outer segments, mutations in *Rhodopsin* can also affect photoreceptor structure. Mutations in *Prph2*, the gene encoding peripherin-2, also cause RP; according to one study of the prevalence of RP-causing mutations, around 8% of RP cases are caused by mutations in this gene [14]. Peripherin-2 is a glycoprotein localising to the outer segments of both rods and cones, providing structural stability to both types of photoreceptor. The first mutations in *Prhp2* were found in patients with RP7, and were shown to be deletions of cysteine residues [17], or of a proline at position 219 [18] causing adRP. Subsequently other mutant *Prph2* alleles have been described; patients with a P216L amino acid substitution present with late-onset dominant RP [18], and the deletion of codon 307 is known to cause dominant RP [19]. Because Prph2 forms tetramers that are inserted into the photoreceptor outer segment, the effect of many of these mutations are dominant-negative; although some wild-type protein is produced, the reduced ability to form functional tetramers results in the integrity of photoreceptor outer segments being compromised, leading to apoptotic cell death. In addition to these cases of monogenic RP, patients carrying mutant alleles for both *Prph2* and another outer segment-specific gene, *ROM1,* suffer from digenic retinitis pigmentosa [20]. Another gene involved with outer segment disc morphogenesis, *RP1*, is mutated in autosomal dominant RP [21]. RP1 is located in the axoneme that forms part of the connecting cilium that links inner and outer segments in photoreceptors [22]. RP1 protein is thought to stabilise nascent outer segments; hence, the absence of RP1 is believed to cause a failure of outer segment formation, leading to apoptotic cell death. RP10, a sub-class of autosomal dominant retinitis pigmentosa, has recently been shown to be caused by mutations in *IMPDH1*. Interestingly, this gene is expressed in a variety of tissues, including spleen, lung, ovary and testis, liver and the eye. Despite this patients with *IMPDH1* mutations only present with retinal degeneration. *IMPDH1* catalyses a key intermediary step in the synthesis of GTP, and it is thought that photoreceptors are particularly susceptible to disruptions in guanine nucleotide

synthesis due to their reliance on GTP for the activation of transducin during phototransduction. Low levels of GTP, due to *IMPDH1* mutations, prevent the retinal phosphodiesterase (PDE) from hydrolysing cGMP to 5' GMP, which in turn leads to a constant influx of calcium ions through voltage-gated ion channels and eventually to cell death. Although patients with mutations in this gene present with early-onset, severe retinopathy, mice lacking *Imdph1* show progressive loss of retinal function without concomitant loss of photoreceptor cells [23]. Deletions, frameshifts and splice-site mutations in *Prpf31* have also been shown to cause adRP with incomplete penetrance [24]. Prpf31 is a protein homologous to a yeast pre-mRNA splicing factor, and is ubiquitously-expressed. *Prpf31* is one of several genes (including *MERTK*, discussed below, and the splicing factor genes *hPrp3* and *Prpc8* that are both implicated in retinitis pigmentosa), that are expressed in other tissues as well as in the eye, but which only give rise to ocular phenotype when mutated. There is currently no evidence that *Prpf31*, or *hPrp3* and *Prpc8*, are involved in splicing of retina-specific, alternatively-spliced isoforms of genes, which could have explained how mutations in these genes only affect photoreceptors. Rather, it is proposed that photoreceptors are highly sensitive to mutations such as those in genes encoding splicing factors due to their high rate of protein synthesis, which is a result of the daily regeneration of outer segment disks [25].

Other genes mutated in adRP include those encoding transcription factors required for photoreceptor development, such as *CRX* and *Nrl*. These are both genes that encode transcription factors that are required for early photoreceptor development, acting together to drive transcription of photoreceptor-specific genes such as *RHODOPSIN* [26-28]. An A→G substitution at position 121 of *CRX* was shown to cause adRP in one family [29], while other mutations in the same gene cause different forms of RP, as discussed below. A Ser→Thr amino acid substitution at position 50 in the NRL protein, caused by a T→A base change in *Nrl*, also causes adRP; this mutation disrupts the transactivation domain of NRL, resulting in inappropriately high transcription of downstream genes, including *RHODOPSIN* [27]. Finally, it has recently been demonstrated that a gene expressed exclusively outside the neuroretina is involved in dominant RP. A R14W substitution in the *carbonic anhydrase IV* gene (*CA4*), expressed in the choriocapilaris, is responsible for RP17 [30]. The authors of this study postulate that incorrectly folded CA4 protein causes the death of epithelial

cells that lead to a disruption of the blood supply to the retina. A subsequent study suggested, however, that a disruption in the regulation of pH is a more plausible mechanism for photoreceptor cell death following on from mutations in *CA4* [31].

1.6.2 Autosomal recessive RP and Leber Congenital Amaurosis (LCA)

Autosomal recessive retinitis pigmentosa (arRP) results from mutations in many different genes, summarised in **Table 1.1**. In general patients with recessive RP present with severe symptoms which in many cases have an early onset. This is most likely due to the fact that most recessive disease segregates with homozygous mutations in genes, often leading to a total absence of the protein encoded; as discussed below, this has severe consequences for photoreceptors that often manifest as a lack of vision from birth and a rapid loss of photoreceptors. Photoreceptor- and RPE-specific genes are known to be mutated in arRP. Cases of autosomal recessive RP have been shown to segregate with several mutations in the α- [32] and β- [33] subunits of the phosphodiesterase enzyme that cleaves cGMP; this prevents the hydrolysis of cGMP, leaving cGMP-gated ion channels permanently open which leads to cell death by calcium overload. Also, some cases of RP are attributed to premature stop codons within the gene encoding the α-subunit of cGMP-gated calcium channels [34], and to various missense mutations in gene encoding the β-subunit [35]. The gene encoding tubby-like protein-1 (TULP-1), a photoreceptor-specific gene that is a member of the *tubby* family of genes [36,37] has also been shown to be mutated in arRP [38,39], and its homologue *tub* causes syndromic retinal degeneration, deafness and obesity when mutated in $tub^{-/-}$ mice [40]. Other photoreceptor-specific genes are mutated in more severe forms of recessive RP, as discussed below. Mutations in the *Mertk* gene, expressed in the RPE, cause early-onset autosomal recessive RP [41]. Patients present with nyctalopia in their early teens and only a small central portion of visual field remaining by their thirties; as such this represents one of the most severe forms of recessive RP. *Mertk* is known to be expressed in kidney, liver, nerve ganglia in the brain and phagocytic cells such as macrophages; despite this wide expression profile, patients with mutations in this gene only present with ocular symptoms.

The most severe recessive retinitis pigmentosa cases are described as Leber congenital amaurosis (LCA), a sub-category of arRP that is characterised by severe visual loss in the first decade of life, and patients have little or no retinal function in terms of electrophysiological response to light. As LCA progresses patients develop the narrowing of retinal blood vessels and pigmentary changes in the RPE seen in other forms of RP, but it is the severe, early-onset visual loss and undetectable ERG accompanied by largely normal fundus appearance that is characteristic of LCA [42]. Many RPE-specific genes encoding proteins that form part of the visual cycle, whereby the 11-*cis* retinal moiety of the photoreceptor visual pigment is regenerated in the RPE, are known be mutated in LCA. Mutations such as those in the *RLBP1* gene, which encodes cellular retinaldehyde binding protein (CRALBP) [43], and in the genes coding for proteins that participate in the conversion of all-*trans* retinol to 11-*cis* retinal such as LRAT, [44] cause LCA. Mutations in the *RPE65* gene are also associated with LCA [45]. As discussed in **Chapter 1.12,** patients with mutations in *RPE65* will be the first to receive AAV-mediated gene therapy as part of a clinical trial to treat LCA. Mutations in photoreceptor-specific genes that lead to LCA have been identified, such as those in *Aipl1* [46], which encodes a chaperone protein expressed exclusively in rod cells [47]. In addition, mutations in the *Gucy2d* gene, which encodes the photoreceptor-specific enzyme that catalyses cGMP synthesis (RetGC) also cause LCA [48]; a missense mutation and two single-base deletions leading to frameshifts were shown to segregate with disease in patients with early onset visual loss and absent ERG. Interestingly, missense mutations elsewhere in the same gene cause dominant cone-rod dystrophy; E837D and R838C amino acid substitutions cause a early-onset loss of central visual acuity, a 'bull's eye' maculopathy followed by peripheral fundus abnormalities, and cone ERG ablation by 7 years of age followed by a decline in rod function by the fourth decade of life [49]. It is proposed that the LCA-causing mutations in *Gucy2d* affect the catalytic domain of the enzyme encoded by this gene, causing recessive retinal degeneration; the E837D and R838C substitutions occur in a putative dimerisation domain, which could interfere with the formation of functional dimmers of RetGC-1 and act as dominant-negative alleles. LCA is also caused by mutations in *RPGRIP*. Following the description of mutations in the gene encoding the retinitis pigmentosa GTPase regulator (*RPGR*) that cause X-linked RP (**Chapter 1.6.3**), the role of the photoreceptor connecting cilium in RP was further elucidated when mutations in

RPGRIP were described. Initially identified through yeast two-hybrid screening for proteins that associate with RPGR [50,51], *RPGRIP* is mutated in patients with LCA [52,53] and was elegantly shown to be the primary resident of the photoreceptor connecting cilium, anchoring RPGR to the cilium; RPGR is absent from the cilia of *RPGRIP*$^{-/-}$ mice, but not the other way around, suggesting RPGR depends on RPGRIP for its correct sub-cellular localisation [54,55].

1.6.3 X-linked retinitis pigmentosa

Retinitis pigmentosa can be inherited in an X-linked form, and thus far mutations in two genes have been identified as causing X-linked RP. The X-RP2 locus was first identified through restriction polymorphism analysis of X-linked RP patients [56], and was subsequently shown to encode a 350 amino acid protein that is expressed in spleen, liver, lung, kidney and photoreceptors [57]; insertion of an L1 retrotransposon element, amino acid substitutions and frameshifts leading to premature truncation of RP2 protein segregate with disease. Mutations in *RPGR*, which encodes an RCC1-like guanosine exchange factor, have been shown to be mutated in X-RP3, the most prevalent form of X-linked retinitis pigmentosa [58]. RPGR was subsequently shown to be an integral part of the photoreceptor protein transport mechanism in mice, through its interaction with the gamma sub-unit of the photoreceptor phosphodiesterase enzyme [59]; its precise function in other mammalian species remains uncertain, although RPGR is located in the connecting cilium in human and in mouse, suggesting the human RPGR has a similar function to its murine orthologue. RPGR is expressed in many cell types, including in the kidney, testis, spleen and liver [60]. ORF 15, a splice variant of *RPGR* preferentially expressed in the retina, appears to be a 'mutation hot-spot' in which many disease-causing mutations reside [60]; this alternatively spliced isoform is created by the skipping of a splice site that causes the extension of exon 15 by translation of intron 15. Subsequently other mutations in *RPGR* have been shown to cause a form of X-linked cone dystrophy (COD1) [61].

The genes responsible for inherited retinal degenerations have been identified in patients through linkage studies and positional cloning strategies, and have provided some insight into the pathology of the various sub-types of retinitis pigmentosa. Both naturally occurring and genetically modified animal models have been used to better understand the complex molecular events leading from mutation of the gene described above to the death of photoreceptors.

1.6.4 Stationary night blindness

Stationary night blindness is caused by a variety of mutations in photoreceptor-specific genes that cause a disruption in function, without the concomitant death of photoreceptors seen in RP. Many cases of autosomal dominant CSNB are caused by an amino acid substitution mutation in the rod-specific *GNAT1* gene [62], whereas several mutations in a calcium channel found in photoreceptors cause X-linked CNSB [63]. Mutations in *PDE6β* also lead to CSNB [64]. The putative mechanisms behind why these mutations lead specifically to stationary disease, as opposed to progressive retinal degeneration seen in RP, are discussed in **Chapter 1.9**.

1.7 Animal models of retinal degeneration

Several animal models of disease show genetic and biological similarity to various forms of RP and have been invaluable in developing gene therapy strategies. These models are generally well-characterised and usually better-understood than their human equivalents in terms of their molecular pathology. Although rodent and large animal retinae differ from human retinae in many aspects, animal models still serve as valid systems in which mechanisms of and treatments for disease can be studied. One major difference between rodent and human retinae is the lack of an anatomically defined macula, or indeed any cone-rich region in the retina. Many animal models of disease differ in some aspects from their human counterparts, and hence the study of therapeutic strategies needs to account for these differences before

conclusions from animal studies can be applied to clinical disease. Thus mutations leading to macular degeneration may be harder to study in rodents. Animal models have recently been developed that do mimic some features of AMD; laser-induced focal disruption of Bruch's membrane leads to choroidal neovascularisation in mice that resembles CNV seen in AMD patients [65], as does ectopic expression of hPK1 [66]. However, these models only reflect individual pathological processes found in AMD, and do so in isolation from the other features of disease seen in humans. Mice with targeted disruption of the gene encoding either macrophage chemoattractant protein-1 (MCP1) or its cognate receptor, C-C chemokine receptor-2 (Ccr-2), do show drusen and lipofuscin accumulation from 15 months of age onwards, thus resembling many features of AMD [67]. These knock-out mice represent the most accurate models of and AMD-like phenotype; nonetheless, as these features of AMD only develop in very old mice, and with an apparent inconsistent penetrance, they are difficult to study as a disease paradigm. As such, it is easier to study retinitis pigmentosa in animal models, as many features of clinical disease are modelled in rodents and large animals.

As well as the animal models that mimic features of AMD (above) and those that reflect the pathology seen in RP (below), animal models with mutant CSNB-causing alleles have also been described. Mutations in the murine orthologues of *GNAT1*, *CACNAF1* and *PDE6β* all cause loss of rod-cell function without concomitant photoreceptor degeneration, and the putative mechanisms behind this are discussed in **Chapter** 9.

1.7.1 Naturally-occurring animal models of retinal degeneration

Genes encoding enzymes of the phototransduction cascade, structural proteins and proteins involved in transport of pigments to photoreceptor outer segments are all found to be mutated in various animal models of RP [4,68]. The first naturally occurring murine model of retinal degeneration to be described was the retinal degeneration (*rd*) mouse [69], which was subsequently shown to have an early onset retinal phenotype due to an insertional mutation in the *Pde6b* gene [70]. This encodes the β-subunit of the PDE enzyme, which hydrolyses cGMP as part of the

phototransduction pathway (see **Chapter 1.4**); the elevated levels of cGMP in the mutant lead to continuous influx of calcium ions through the cGMP- and voltage-gated Ca^{2+} channels, and this leads to apoptotic cell death in photoreceptor cells due to calcium overload [71]. In contrast to human arRP, the *rd* mouse has an early onset rapid degeneration, with rod cell loss complete by post-natal day 20 [72]; RP patients with mutations in β-PDE (the human orthologue of *Pde6b*) present later in life, well into their third decade. This discrepancy may well be due to the fact that whilst the disease-causing mutation in the *rd1* mouse is a null mutation resulting in no functional protein being produced, a large proportion of patients with *PDE6β* mutations have missense mutations affecting the catalytic domain of the mature protein, resulting in reduced but not abolished activity [70,73].

Recently the $Rpe65^{Rd12/Rd12}$ mouse was characterised, which is a naturally-occurring model for LCA caused by a homozygous nonsense mutation in the *Rpe65* gene [74]. The $Rpe65^{Rd12/Rd12}$ mouse has distinctive white dots visible on fundus examination at five months of age, and severely reduced ERG b-wave amplitudes (around 50 μV) at one month of age. The two most widely-studied naturally-occurring rodent models of RP, the $Prph2^{Rd2/Rd2}$ mouse and the RCS rat, are discussed in more detail below. There are also large-animal models of human retinal degeneration. Mutations in the canine equivalent of the *Pde6b* gene cause retinal degeneration in a dog model of RP, wherein Irish setter dogs have an early-onset retinal degeneration phenotype that segregates with the locus designated rod-cone dysplasia 1 (*rcd1*). This was shown to be a mutant allele of the gene encoding the β-subunit of canine rod cGMP phosphodiesterase; a stop codon created by a single base substitution at codon 807 leads to a loss of functional PDE-β protein [75], which is analogous to the *rd1* mouse described above. The *rcd1* dog is another model used for investigating the progression of disease and possible treatments for RP, and has an early onset of retinal degeneration (around 25 days after birth) followed by loss of photoreceptors over one year [75]. A further naturally-occurring large animal model of RP is caused by a T4R mutation in *rhodopsin* found in English mastiff dogs, which causes abnormal photorecovery following light excitation [76]; this phenotype is similar to that seen in patients with a T4K amino acid substitution, and thus this naturally occurring dog model of RP may prove useful in the evaluation of therapeutic strategies for this form of RP. A canine model for X-linked RP has also been described, known as X-linked

progressive retinal atrophy (XLPRA); Siberian husky dogs suffer from an X-linked rod-cone dystrophy that was initially mapped to a region of the X chromosome that has homology to the human RP3 locus [77]. The two sub-categories of this canine retinal dystrophy, XLPRA1 and XLPRA2, were subsequently shown to be caused by two different mutations in the *RPGR* gene [78]. Deletion of five nucleotides in exon ORF15 of *RPGR*, leading to premature stop codon that truncates RPGR ORF15 by 230 amino acids, causes XLPRA1. This form of XLPRA is characterised by normal photoreceptor development and onset of ERG abnormalities and photoreceptor loss at one year of age. XLPRA2 was shown to segregate with a two base-pair deletion further downstream within exon ORF15, which leads to the inclusion of 34 extra basic amino acids in RPGR and causes photoreceptors to develop abnormally; XLPRA2 retinae have greatly reduced ERG response from an early age, and a rapid loss of photoreceptors such that 50% of the ONL is lost within seven months of life [78]. Thus, rodents and dogs that have naturally-occurring mutations have been extensively studied in order to understand the mechanisms underlying human retinitis pigmentosa; transgenic technology has also been used in rats and mice to study the pathology of RP, as discussed below.

1.7.2 Light-induced, transgenic and knock-out rodent models of retinal degeneration

Photoreceptor death has been modelled in rodents by using constant light exposure (or exposure to short bursts of high levels of light) to induce damage. There are advantages to inducing photoreceptor death in this manner; the cells die by apoptosis so some insights into mechanisms of photoreceptor death can be gained be studying light-damage models. Also the photoreceptor death is acute and rapid, with exposure to 2 hours of 13,000 *lux* light causing complete loss of photoreceptors in 10 days, such that therapeutic strategies take less time to evaluate than in some genetic models of disease. Nonetheless, there are major discrepancies between such light-induced models of cell death and RP seen in genetic models of disease and in the clinic. Firstly, although photoreceptors die by apoptosis, the mechanisms that trigger this programmed cell death (see **Chapter 1.9**) may be very different to those in

genetic models of RP, which means treatment strategies that show efficacy in these acute models of disease may not be appropriate to clinically-relevant models. Also, the acute nature of photoreceptor death in these models means that there is a very small 'window' in which to intervene with a therapy; often therapies need to be applied as prophylaxis in advance of light-induced damage, an option not available in the clinic. Hence the studies presented here, and the majority of studies into potential therapies for RP, use genetic models of disease.

Transgenic technology, whereby a mouse strain is engineered to harbour a disease-causing allele of a gene implicated in RP, and targeted disruption of endogenous murine genes, have both been used extensively to study the pathology of RP. A mouse harbouring a Q344ter mutation in the *rhodopsin* gene has a naturally occurring stop codon that truncates the rhodopsin protein by five amino acids, resulting in mislocalised rhodopsin and a more latent response to a light stimulus [79]. Rodents expressing transgenes carrying many other mutations in the rhodopsin gene, equivalent to those mutations found in patients, show photoreceptor degeneration [4]. For instance, rats carrying an S334Ter transgene undergo rapid retinal degeneration, losing most of their photoreceptors within three weeks [80]. The proline-to-histidine substitution at position 23 was the first rhodopsin amino acid substitution to be described in patients [13], and rats carrying a P23H *rhodopsin* transgene are perhaps the most widely studied transgenic model of RP. These P23H rats undergo a slower photoreceptor degeneration than the S334Ter rats; rats heterozygous for the P23H opsin allele lose around 50% of their photoreceptors at three months of age [81]. In addition to transgenic rodents carrying mutant versions of *rhodopsin*, other dominant disease alleles have been used to study RP in mice. A mouse strain carrying a *Prph2* transgene that encodes a P216L amino acid substitution has been shown to lose photoreceptors and retinal function in a manner consistent with patients that have the P216L mutation [82]. The mutant *Prph2* allele causes photoreceptor degeneration due to a reduced ability to form functional peripherin-2 tetramers in photoreceptor outer segments. This loss of photoreceptors is dependent on the level of transgene expression in different lines of P216L transgenic mice (high-expressing lines have 2 rows of photoreceptor nuclei in their outer nuclear layer at seven months of age, compared with five rows in lower-expressing lines), and on the genetic background of the mice with respect to the *Prph2* gene (on a $Prph2^{Rd2/+}$ heterozygote background,

mice carrying a P216L *Prph2* transgene have 5 rows of photoreceptor nuclei at three months of age). Knock-out mice, created by targeted disruption of a gene by a selective marker, have also been generated to mimic mutations that lead to RP; for instance, targeted disruption of the *rhodopsin* gene has been used to study RP, with *rhodopsin*$^{-/-}$ mice losing all their photoreceptors within the first three months of life [83]. Mice carrying one functional allele of *rhodopsin* (*rhodopsin*$^{-/+}$) have a normal outer nuclear layer at this time point, although individual rod cells express 50% of the normal level of *rhodopsin* [84]. Mice lacking the *Tulp1* gene serve as a model of recessive RP [85]. In *tulp1*$^{-/-}$ mice there is mislocalisation of rod- and cone-opsins, accumulation of vesicles in the inter-photoreceptor matrix, and photoreceptor degeneration that reaches end-stage at 5 months of age. Mice lacking *RPGR* and *RPGRIP*, which encode proteins found in photoreceptor connecting cilia, serve as models for X-linked and LCA respectively [54,86]. *RPGR*$^{-/-}$ mice show normal photoreceptor development, followed by cone opsin mislocalisation to inner segments and a reduction in amount of rhodopsin present in rod outer segments. Functionally the lack of RPGR protein leads to a 25% reduction in rod-specific ERG at 6 months of age, and a concomitant loss of cone function, and structurally the outer nuclear layer is reduced by two rows of photoreceptor nuclei at this time point [86]. Mice lacking *RPGRIP* show a much more severe phenotype than those lacking *RPGR*; *RPGRIP*$^{-/-}$ photoreceptors have abnormal outer segments and pyknotic nuclei from a very early age (PND 15), and photoreceptor loss is complete by three months of age [54]. RPGR protein was shown to be absent in connecting cilia of *RPGRIP*$^{-/-}$ mice, but RPGRIP is present as normal in mice lacking *RPGR*; this indicates that RPGRIP localises to the connecting cilium first, recruiting RPGR subsequently, and that a lack of RPGRIP gives rise to a more severe, early-onset phenotype which is analogous to clinical findings [54].

In selecting models of retinal degeneration in which to test therapeutic strategies, several considerations need to be made. Firstly, models which resemble human disease, both in terms of the mutation that causes disease and in the pathology that follows, are desirable in order that any successful treatment could be considered for use in the clinic. Secondly, a model that shows an intermediate rate of photoreceptor degeneration is ideal; models that lose their photoreceptors slowly enough to allow therapeutic intervention (as opposed to the *rd1* mouse, for instance), and yet not so

slowly that evaluating the efficacy of the therapy becomes difficult (e.g. *RPGR*$^{-/-}$ mice). Thirdly, a rodent model of retinal degeneration that has an absence of a given gene product, as opposed to those caused by dominant negative alleles, may prove more amenable to treatment as discussed in **Chapter 1.13**. The studies presented here focus on two models of retinitis pigmentosa that, by virtue of their relevance to disease found in humans, the favourable kinetics of photoreceptor loss and the homozygous null nature of the mutations, are invaluable tools in elucidating the mechanisms involved in retinal degeneration.

1.8 Biology of the *Prph2*$^{Rd2/Rd2}$ mouse and RCS rat

Formerly known as the retinal degeneration slow (rds) mouse, the *Prph2*$^{Rd2/Rd2}$ mouse has much in common with cases of RP in humans, with an intermediate rate of photoreceptor cell loss compared with other models of disease [68]; degeneration proceeds slower than models such as the *rd1* mouse (in which one layer of photoreceptor cells remain at three weeks of age), but faster than models such as *RPGR*$^{-/-}$ mice – in *Prph2*$^{Rd2/Rd2}$ mice the ONL is reduced by 50% by two months of age, and continues to atrophy over the next six months. It is not clear why different models of RP show such variable rates of photoreceptor loss, although some putative mechanisms are discussed in **Chapter 9**. However, one major difference between this naturally occurring model and human disease is that the *Prph2*$^{Rd2/Rd2}$ mouse has negligible rod function and greatly reduced cone function from birth (see below), whereas many patients retain scotopic vision until the fifth or sixth decade of life. While the inheritance mode in this mouse model is semi-dominant, the kinetics of photoreceptor degeneration resembles that seen in dominant RP in humans. Disease is caused by an insertional mutation in the Peripherin (Prph2) gene that encodes peripherin-2, a structural protein found in the outer segments of photoreceptors [87]. Peripherin-2 is a major structural component of the membranous disks within the outer segments that house the visual pigment in both cones and rods. Failure to produce these disks, due to the null mutation in the *Prph2* gene, causes absence of outer segments from birth (see **Fig. 1.11**). As outer segments are not formed, and the rhodopsin pigment cannot be correctly localised to the outer segment,

phototransduction takes place at greatly reduced levels than in wild-type mice. The mechanisms that link the absence of outer segments to photoreceptor cell death are unclear. Previous studies have established that cell death in the *Prph2*$^{Rd2/Rd2}$ mouse (and other rodent models of retinitis pigmentosa) occurs by apoptosis, and that in the case of the *Prph2*$^{Rd2/Rd2}$ mouse this programmed death peaks at post-natal day 18 [72]. The particular kinetics of photoreceptor cell death in the *Prph2*$^{Rd2/Rd2}$ mouse model has implications for the type of treatment that might be tested, and this will be discussed in more detail in **Chapter 1.9**.

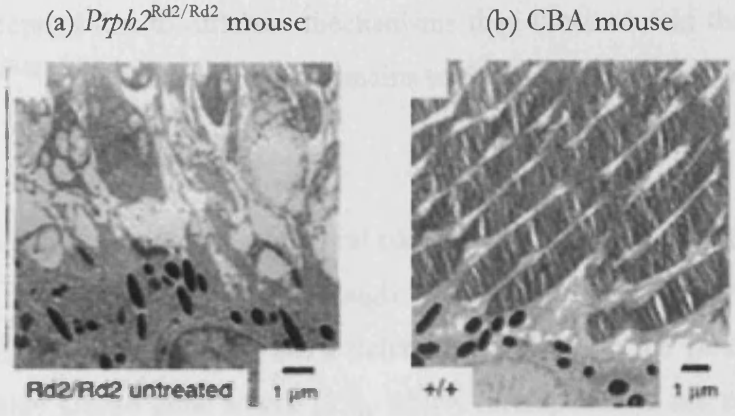


Fig. 1.11: Outer segments in *Prph2*$^{Rd2/Rd2}$ and wild-type CBA mice; mice homozygous for a null mutation in *Prph2* do not develop outer segments and have disorganised inner segments (a) when compared to wild-type mice (b).

In the *Prph2*$^{Rd2/Rd2}$ mouse some residual electrophysiological activity is detectable in terms of a scotopic ERG b-wave in the first weeks after birth. In the absence of outer segments, the rhodopsin produced by rods and cones is localised in the connecting cilium. This reduced, mislocalised pigment does give some activation of phototransduction, which provides enough electrical activity to generate a small response to a light stimulus. It remains unclear, however, whether *Prph2*$^{Rd2/Rd2}$ mice have residual vision, even before the large scale death of their photoreceptors. The earliest recordings of young *Prph2*$^{Rd2/Rd2}$ mice show a b-wave amplitude of 40 µV at a rod-specific stimulus intensity. This functional deficiency worsens over the first few weeks after birth, with no detectable b-wave present at six months of age [88].

Mice heterozygous for the insertional mutation in *Prph2* (*Prph2*$^{Rd2/+}$) also have an RP-like phenotype, although the kinetics of retinal degeneration is altered from that seen in homozygous mice. *Prph2*$^{Rd2/+}$ heterozygotes have largely normal retinal development, although electron microscopy reveals 'whorls' in the photoreceptor outer segments caused by a reduction in the amount of Peripherin-2 protein being produced. *Prph2*$^{Rd2/+}$ mice retain largely wild-type ERG responses into their third month, following which there is a gradual decline in b-wave amplitude. This is reflected in the relatively slow progression of apoptosis in the rod cells, with 50% of the outer nuclear layer lost by eighteen months in heterozygote mice as opposed to two months in the *Prph2*$^{Rd2/Rd2}$ homozygote [89]. It may be that *Prph2*$^{Rd2/+}$ mice lose their photoreceptors due to different mechanisms than those seen in the homozygous null *Prph2*$^{Rd2/Rd2}$ mouse, although this remains to be determined experimentally.

The Royal College of Surgeons (RCS) rat completely loses its outer nuclear layer by around three months of age, even though the mutated gene is not expressed in photoreceptor cells. The RCS rat has a deletion of 409 bp in the gene encoding the receptor tyrosine kinase gene Mertk [93], which corresponds to the second exon of that gene; expressed in the retinal pigment epithelium (as well as monocytes), Mertk encodes a proto-oncogene, constitutively active mutants of which are implicated in tumourigenesis [90]. Lack of Mertk protein prevents the RPE cells from phagocytosing the outer segments of photoreceptors [95], which are shed as part of the circadian cycle and accumulate as debris in the sub-retinal space. This prevents oxygen and nutrients reaching the photoreceptor cells, leading to complete loss of cells in the outer nuclear layer by three months after birth.

There are several advantages in using the *Prph2*$^{Rd2/Rd2}$ mouse and the RCS rat as models for retinal gene therapy. Primarily, as the *Prph2*$^{Rd2/Rd2}$ mouse is one of the best characterised models of RP in terms of its biology, some of the most extensive gene therapy work to date involves this strain of mice. The rate of degeneration in both of these rodent models is favourable; treatments can be assessed relatively quickly after administration as the rate of ERG loss and of photoreceptor loss is fast enough to determine efficacy. Moreover, the rate of degeneration is somewhat slower

than in the rd mouse or light-induced models of disease, and this in turn allows for a time window in which treatments can be applied and assessed before retinal structure and function is ablated completely. In order that such gene therapy applications are successful, it is necessary to determine the mechanisms underlying apoptotic photoreceptor loss in models of retinal degeneration, as discussed below.

1.9 Mechanisms of photoreceptor death in inherited retinal degeneration

The mutations discussed above have been associated with retinitis pigmentosa through genetic linkage studies in humans, and are known to cause retinal degeneration through studying animal models of disease. It has also been previously shown that the cell death seen in these models is caused by apoptosis [72]. However, little is known about the link between the genetic defects know to cause disease in models of RP, and the death of retinal cells by programmed cell death. Several hypotheses on the mechanisms of photoreceptor death have been proposed, with many different pathways discussed. By understanding the way in which mutations in the genes mentioned above cause the apoptosis seen in RP, it may be possible to intervene in the appropriate pathways, slowing or even preventing this cell death.

1.9.1 Overview of apoptotic signalling

Programmed cell death, or apoptosis, is an essential process required to control cell numbers in multicellular organisms, and is involved in normal embryological development. However apoptosis is a common consequence of disparate disease-causing mutations, and this controlled cell death leads to loss of photoreceptors in retinitis pigmentosa. Apoptosis is mediated by a signalling cascade involving the activation of caspase enzymes by proteolytic cleavage. Synthesised as pro-enzymes (pro-caspases), caspases cleave their downstream target molecules at specific aspartic

acid residues, using a critical cysteine residue in their active site (**Fig. 1.12**). The initial stimulus for the activation of this caspase cascade can be intracellular (i.e. in response to damage within the cell) or extracellular (i.e. in response to signalling from other cells). The intracellular triggers for apoptosis are varied, and centre on the mitochondrion; this pathway is discussed in more detail below. In the case of extracellular triggers of apoptosis, receptor-mediated signalling initiates the cleavage of activator caspases by causing the aggregation of adaptor proteins, molecules that recruit specific pro-caspases into a multi-protein complex with proteolytic activity. The receptors used to trigger apoptosis share common intracellular domains, known as death domains, which bind to adaptor proteins only when their ligand is bound to their extracellular domain; for example, the Fas ligand-mediated activation of the Fas receptor mediates Caspase-8 cleavage through its intracellular death domain during apoptosis of T-cells [91].

Aggregated pro-caspases such as Caspase-8 undergo 'mutual cleavage' (i.e. each Caspase-8 molecule cleaves another Caspase-8 molecule in the aggregate), rendering many active molecules of this activator caspase. These active enzymes then cleave their specific target caspases, stimulating their proteolytic activity. This caspase activation pathway terminates in fragmentation of the nuclear envelope, DNAse digestion of chromosomal DNA and degradation of cellular lipids in a systematic dismantling of the cell (**Fig. 1.13**).

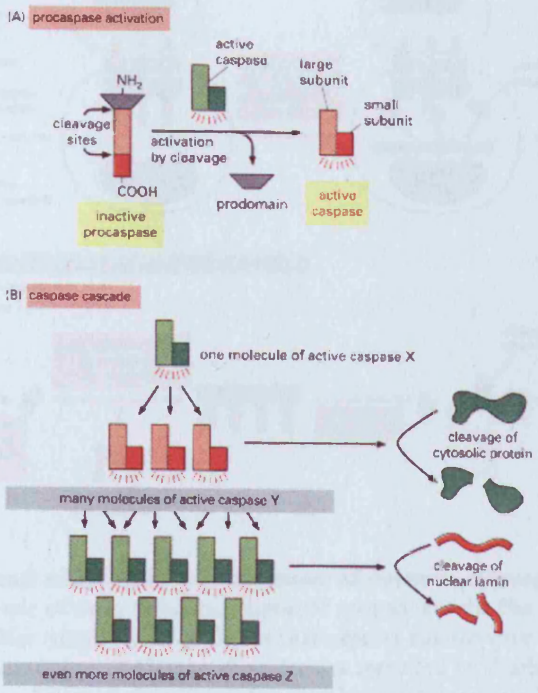


Fig. 1.12: Schematic diagram showing activation of caspase activity; Following cleavage by an activator caspase, an inactive procaspase molecule becomes and active caspase (A), and is able to cleave its downstream target caspases to initiate a proteolytic cascade that terminates in breakdown of nuclear laminin proteins, degradation of cytosolic proteins and cell death (B).

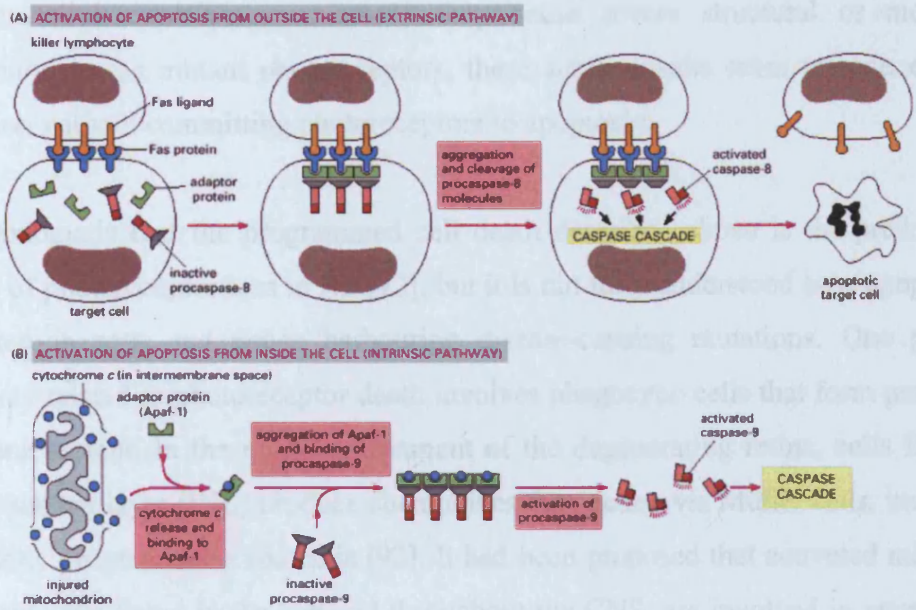


Fig. 1.13: Intra- and extra-cellular stimulation of caspase cleavage cascade during apoptosis; an example of extra-cellular initiation of apoptosis is the Fas-ligand mediated cell death induced by killer lymphocytes of T-cells that express Fas-receptor (A); binding of Fas-ligand leads to accumulation of Caspase 8 molecules recruited by death-domain binding of adaptor molecules, and mutual cleavage within the Caspase 8 aggregate leads to initiation of a caspase cascade. Intracellular stimuli can also initiate apoptosis, and this commonly involves injury to the mitochondria (B); injured mitochondria release Cytochrome C, which bind to the activator molecule Apaf-1. The subsequent activation of procaspase 9 leads to the proteolytic caspase cascade described previously.

1.9.2 Apoptosis in inherited retinal dystrophies

Before considering the ways in which RP-causing mutations cause cell death, it is worth discussing the *failure* of CSNB-causing mutations to cause photoreceptor death. As detailed in **Chapter 1.6.4**, mutations in (amongst others) *GNAT1, CACNAF1* and *PDE6β* lead to disruption in photoreceptor function, without causing the mutant cells to die. This may be related to how the mutations affect photoreceptor function. It appears that mutations leading to CSNB modify photoreceptor function in a more subtle manner than those leading RP, either by altering the synaptic signalling between photoreceptors and bipolar cells (in the case of *CACNAF1* mutations, which lead to intact photoreceptor function but reduced ERG b-wave and hence reduced

higher visual function), or by altering the regulatory mechanisms in the phototransduction cascade (i.e. point mutations in *GNAT1* and/or *PDE6β*). As neither type of CSNB-causing mutation would be predicted to result in a prolonged reduction in intracellular calcium, nor would they cause severe structural or metabolic disruption to the mutant photoreceptors, these subtle insults seem to reduce visual function without committing photoreceptors to apoptosis.

It is axiomatic that the programmed cell death described above is the predominant cause of photoreceptor loss in RP [72], but it is not fully understood how apoptosis is initiated in rods and cones harbouring disease-causing mutations. One possible pathway related to photoreceptor death involves phagocytic cells that form part of the immune system. In the microenvironment of the degenerating retina, cells from the inner nuclear layer (INL) produce chemokines that, acting via Müller cells, induce the apoptotic programme in rod cells [92]. It had been proposed that activated microglia, myeloid cells found in the eye and throughout the CNS, are involved in apoptosis in the outer nuclear layer of dystrophic retinae; because numbers of activated microglia peak at the same time as apoptosis peaks in the RCS rat, an hypothesis emerged suggesting that microglia play an active role in photoreceptor cell death. However, recent work in the *Prph2*$^{Rd2/Rd2}$ mouse shows that although microglia are present in the degenerating retina, neither their gene expression profile nor their temporal distribution supports this theory as microglial presence peaked 5 days after apoptosis had done so and the microglia were also iNOS-negative [93], suggesting the absence of cytotoxic signalling molecules that could kill photoreceptors.

Another theory of cell death in RP concerns the constitutive activation of the phototransduction cascade, caused by aberrant expression of genes required to prevent over-stimulation of photoreceptors. Fain and Lisman first proposed a mechanism by which photoreceptors die in response to vitamin A deprivation and inherited RP mutations [94]. They suggested that the cell death seen in these circumstances could be similar to that seen in light damage models of RP. In such models, retinae exposed to either short bursts of high-intensity light or prolonged low intensity light suffer loss of photoreceptors. Their 'equivalent light hypothesis' suggests that many of the mutations that cause RP, as well as dietary vitamin A deprivation, may cause constitutive stimulation of the phototransduction cascade, resulting in signals being

sent to the inner retina that are 'equivalent' to those sent in response to the detection of incident light. It has been proposed this may lead to cytotoxicity by two mechanisms; one in which insufficient calcium influx leads to cell death (see below), and another whereby the post-synaptic bipolar cells, acting as if constantly exposed to light, initiate signalling events that lead to photoreceptor cell death. Evidence has since emerged to suggest that this may be a major contributing factor to the degeneration seen in some forms RP. For instance, data *from CNGB1*$^{-/-}$ mice, which produce no β-subunit of the cGMP-gated calcium channels, support the theory that continuous activation of phototransduction leads to cell death. Consider that light capture by rhodopsin leads to activation of PDE via transducin, and that this leads to closure of the Ca^{2+} channels, hyperpolarising the cell and preventing the glutamate release that forms the constitutive 'dark signal'. Disruption of the *CNGB1* gene that leads to lack of CNGB1 protein results in calcium influx being permanently prevented, because CNGB1 is required to correctly target the CNGA1 protein to the rod plasma membrane and to form functional CNG heterotetramers with α-subunits. Due to the lack of functional cGMP-gated calcium channels at the plasma membrane, calcium is no longer transported into the cell to maintain a depolarised 'ground state' and there is a permanent state of hyperpolarisation in the photoreceptor inner segment; glutamate is never released from the photoreceptors, and the bipolar cells of the inner retina respond as though they are receiving signals from light-activated rods all the time.

Evidence from other models of RP also supports the equivalent light hypothesis. Some mutant forms of rhodopsin are known to constitutively activate transducin, independent of excitation by photons of light [95,96]. There is some difficulty in directly assaying the constitutive activity of such mutants *in vivo*, but if transducin is activated even in the absence of light the inner retina would receive an equivalent light signal as before. Mutations in the retinal guanylyl cyclase enzyme, RetGC, have also been described [49]. The expected consequence of this would be the lack of cGMP synthesis, leading to permanently closed calcium channels and a signal equivalent to light being sent to bipolar cells and beyond in the visual pathway. In addition, a study using mice that lack functional arrestin protein and are defective in the shut-off of rhodopsin signalling showed that activation of phototransduction by normal levels of light can cause degeneration of the outer nuclear layer over a period

of one year; this is because the phototransduction cascade is not correctly shut off, supporting the equivalent light hypothesis [97]. Perhaps the most convincing evidence to support the equivalent light hypothesis comes from a transgenic mouse model of Leber's congenital amaurosis (LCA). Woodruff and colleagues showed that a null mutation in the *Rpe65* gene, known to cause LCA by disrupting 11-*cis* retinal reconstitution from recycled all-*trans*-retinol, causes degeneration of photoreceptors in a manner consistent with the equivalent light hypothesis; photoreceptors from *Rpe65*$^{-/-}$ mice have reduced circulating current in response to light stimulation, reduced light sensitivity, and accelerated turn-off of photoresponse [98]. Moreover, they provide direct evidence that the lack of RPE65 protein causes photoreceptor death through accumulation of opsin that is not bound to 11-*cis* retinal and the constitutive activation of the phototransduction cascade; by crossing the *Rpe65*$^{-/-}$ mice with mice homozygous for a null mutation in *transducin*, and showing a retardation of cell death, they showed that the *Rpe65* null mutation causes photoreceptors apoptosis mediated by activation of the phototransduction cascade [98]. Thus, new, more direct evidence suggests that a constitutive signal equivalent to that sent on light exposure may cause cell death in RP.

To elaborate upon this hypothesis, the mechanisms through which such constitutive activation of the phototransduction cascade causes apoptosis need to be elucidated. Two possibilities arise; one is a cell-autonomous, intracellular pathway to cell death, another implicates other cell types associated with apoptotic photoreceptors. Intracellular responses from within cells that harbour mutations leading to an equivalent light signal could be responsible for triggering apoptosis, possibly related to calcium levels within the photoreceptors. The permanent closing of calcium channels, through any of the mechanisms mentioned above, would lead to a drop in intracellular calcium levels. Neuropathology in various systems is associated with *elevated* Ca^{2+} levels, through mitochondrion-mediated caspase activation that follows signalling from glutamate receptors [99]; the degeneration in the *rd* mouse could be caused by elevated intracellular calcium, produced by the constitutively high cGMP levels keeping the Ca^{2+} channels open. However, some studies suggest that neurotoxicity may arise if calcium levels are too low as well. *In vitro* experiments show that the gating of calcium channels, leading to an increase in intracellular Ca^{2+}, protects cultured neurones from apoptosis [100]. Another way in which cells may die

in response to an equivalent light signal could involve secondary messengers being released by cells in the inner retina. Bipolar cells, as well as Müller cells and ganglion cells, secrete neurotrophic factors that promote the survival of photoreceptors under normal circumstances. It may be that on receiving a constant light signal, real or equivalent, these cells alter their gene expression and either secrete factors that trigger an extrinsic apoptotic pathway in the photoreceptors, or stop secreting factors that otherwise promote photoreceptor survival.

It is likely that the evidence supporting the equivalent light theory applies better to certain models and forms of RP than others. In particular, several forms of RP are caused by mutations that give rise to disease mechanisms considerably different from constitutive activation of the phototransduction cascade; mutations – particularly homozygous null mutations – that ablate photoreceptor function totally are likely to cause cell death due to the *abolition* of photoresponse in the rods and cones, as opposed to the constitutive activation of phototransduction proposed in the equivalent light hypothesis. As such these mutations would represent an 'equivalent dark' hypothesis, whereby lack of phototransduction is a characteristic consequence of the gene defect concerned. By understanding the alternative disease mechanisms leading to apoptotic photoreceptor death, therapeutic intervention to prevent the loss of cells in the outer retina may be possible. For instance, healthy photoreceptors are known to die in the presence of mutant cells; this 'by-stander effect' suggests a non cell-autonomous mechanism for cell death, possibly involving secondary cells and their secreted factors [101]. Direct evidence for this by-stander effect comes from chimeric mice generated from albino *Prph2*$^{Rd2/Rd2}$ mice and pigmented mice that were wild-type at the *Prph2* locus; photoreceptors were found to die not only in the regions overlying non-pigmented RPE, but also in regions overlying pigmented RPE, indicating that photoreceptors that are genetically normal die in the presence of photoreceptors carrying RP-causing mutations [102]. The involvement of inner retinal cell types in the death of otherwise healthy photoreceptors has not yet been shown, and this remains a plausible theory of cell death in RP. However, evidence that a by-stander effect mediated by the loss of rods is responsible for the cone cell death has emerged from a series of studies on retinal explants from rod-deficient *rd* mice. The rod-derived cone viability factor (RdCVF) was isolated by screening a cDNA library from a wild-type mouse retina for a secreted factor that enhances the survival of chick

cone cells and cone-rich retinal explants from *rd1* mice in culture [103,104]. This 17 KDa protein is secreted by rods, and, when delivered as a GST-tagged fusion protein into the sub-retinal space of *rd* mice, supports enhanced survival of cones. RdCVF is the first example of a factor that is secreted by rods that appears to be required for cone survival. It is not known how RdCVF acts on cone cells to enhance their survival, but it has been established that removal of the factor from rod-conditioned medium by blocking antibodies abrogates the ability of the conditioned medium to enhance murine cone cell survival in explants [103]. As RdCVF is secreted by rods and acts on the survival of cones, there is at least a component of photoreceptor death that is not cell autonomous; it is as yet unclear whether RdCVF plays a role in photoreceptor death following insults such as continuous light or equivalent genetic mutations, and because many of the experiments described using RdCVF were done *in* vitro using either chick cells of murine retinal explants, it is unclear whether this molecule is relevant to cone survival *in situ* in either the murine or human retina. It is yet to be determined whether gene therapy using this rod-derived survival factor would enhance cone cell survival.

In addition to the effect of rod death on cone survival, another way in which other cell types may be implicated in photoreceptor death is through a change in the expression of survival factors that are secreted by inner retinal cells. It is known that in order to prevent neuronal cells from undergoing apoptosis, glial cells and neurones themselves express many protein growth factors that are neurotrophic – factors that promote the survival of neuronal cells. Such neurotrophic factors are secreted by the cells that neurones grow towards (their target cells), and binding of the neurotrophic factor to its cognate receptor leads to signalling that prevents apoptosis in the neurone, as well as growth of the neurone towards the source of the factor along a concentration gradient [105]. Following the identification of nerve growth factor (NGF), seen as the prototypical neurotrophin, several proteins have been shown to promote neuronal survival in the CNS, including NGF itself [106], brain-derived neurotrophic factor (BDNF) [107], ciliary neurotrophic factor (CNTF) [108], basic fibroblast growth factor (bFGF) [109] and glial cell line-derived neurotrophic factor (GDNF) [110]. These studies demonstrated the potential of neurotrophic molecules in promoting neuronal cell survival; the term neuroprotection refers to this survival effect on neuronal cells exerted by neurotrophic growth factors. Withdrawal of these growth

factors is sufficient to initiate photoreceptor apoptosis, although this may not be the only mechanism involved in retinal degeneration.

In a review of mechanisms involved in apoptosis of photoreceptors in models of RP, Travis proposed that mutations that cause equivalent light signals, and those that disrupt photoreceptor function, may lead to cell death through an increase in oxidative stress [111]. When expression of the anti-apoptotic gene *Bcl-2* was shown to improve photoreceptor survival in *Prph2*$^{Rd2/Rd2}$ mice [112], this notion was given credence. It is proposed that the unusually high density of mitochondria in photoreceptors makes rod and cone cells unduly sensitive to mutations that alter their metabolic requirements. Mutations such as that of the *Peripherin* gene in the *Prph2*$^{Rd2/Rd2}$ mouse lead to photoreceptors being less metabolically active than wild-type photoreceptors. This may lead to an increase in mitochondrial free radical production in the form of reactive oxygen species (ROS). This is because, whilst other tissues are able to modulate their blood supply by vaso-constriction, the choriocapilaris that serves the retina is largely unable to constrict, meaning the same level of oxygen is supplied to photoreceptors despite their altered metabolic state. In many cell types, in both normal processes such as embryological development and in the pathology of various diseases, apoptosis occurs when various stimuli trigger intracellular signalling cascades through mitochondria. The role of mitochondrial responses to oxidative damage in apoptotic cells in general is well-established; the release of Cytochrome-c from the mitochondrion (in response to, amongst other stimuli, oxidative stress) and the subsequent activation of caspase proteins via Apaf-1 is seen as a major control point in the intracellular pathway to programmed cell death [91]. Hence, a rise in mitochondrial ROS through a mutation that causes a reduction in metabolic activity would then lead to increased apoptosis, as ROS are known to induce damage to DNA, lipids and proteins within mitochondria [113,114], leading to mitochondrial inner membrane disruption and subsequent cytochrome-c release.

This receptor-independent response to internal sensing of apoptotic stimuli such as oxidative stress is likely to be a predominant cell death pathway in the *Prph2*$^{Rd2/Rd2}$ mouse, particularly when considered alongside a mathematical model for retinal apoptosis [115]. Clarke *et al.* describe how the probability that any given rod cell will die by apoptosis declines exponentially over time, and that this can only be explained

by a 'one-hit model' of cell death whereby a mutation leaves cells susceptible to a single apoptotic insult. This is in contrast to, for instance, a 'cumulative damage' model for cell death, where a steadily increasing probability of apoptosis would be seen. The authors of this regression analysis study demonstrated that the one-hit model also applies to other animal models of retinal degeneration, including the *nervous* (*nr/nr*) mouse, the *purkinje cell degeneration*(*pcd/pcd*) mouse, and the *rd1* mouse [115]. Following this analysis it has been proposed that the kinetics of apoptosis seen in the $Prph2^{Rd2/Rd2}$ retina, and in other models of retinal degeneration, suggests that mutations affecting the integrity and functional status of outer rod segments leave cells vulnerable to increased oxidative stress, and that this oxidative stress leads to an exponentially decreasing loss of rod cells by programmed cell death.

Within this 'oxidative stress' theory of retinal cell death, the accumulation of reactive oxygen species is crucial; mitochondria in cells with mutant outer segments are more likely to produce free radical species that go on to damage mitochondrial and cellular components. The natural cellular defence against ROS is the manganese superoxide dismutase enzyme, MnSOD; this enzyme is the major mitochondrial member of the superoxide dismutase gene family, and is encoded by the nuclear *SOD2* gene [116]. SOD2 converts ROS to H_2O_2, removing a free radical in the process. Although the half-life of oxygen free radicals *in situ* are very short, making the ROS themselves difficult to quantify, proximate markers of ROS damage such as peroxidated lipids, DNA lesions and protein peroxidation can be measured to assess the involvement of ROS-mediated damage in cells. Considering that $SOD2^{+/-}$ heterozygotes show increased ROS presence and increased oxidative damage to mitochondrial DNA [117], overexpression of *SOD2* in models of RP in which absence or paucity of outer segments is believed to reduce rods' oxygen utilisation should prevent or slow apoptotic death. Conversely, a mouse that is heterozygous for null mutations in both *SOD2* and *Prph2* ($SOD2^{+/-}$ $Prph2^{Rd2/+}$ mice) might produce accelerated apoptotic photoreceptor cell death as shown by thickness of the outer nuclear layer.

1.10 Treatment strategies for RP

1.10.1 Pharmacological treatments for RP

Pharmacological treatments are available for many ocular diseases, and range from systemically to locally administered compounds. However, none of the forms of retinitis pigmentosa that have been discussed thus far benefit from pharmacological interventions. Some RP-like syndromic conditions may be ameliorated by dietary regulation, such as gyrate atrophy of the retina and choroids [118]. However, any improvements seen are reversible, and some reports suggest that certain patients may not respond to treatment at all [119]. Drug therapies have been largely ineffective in animal models of RP. A study in the *rd* mouse suggesting a (transient) amelioration of phenotype following systemic administration of calcium channel blockers [120] was contradicted by another study in the same model [121] whereas oral administration of 9-*cis*-retinal in *Rpe65*$^{-/-}$ mice is thought to mediate therapeutic benefit, albeit only when assessed at short-term time points [122,123] . A more common pharmacological approach, however, involves attempts to prevent photoreceptor cell loss without addressing the underlying causative mutations. As discussed previously, neurotrophic factors exert an anti-apoptotic effect by suppressing pro-apoptotic signalling in various neurones. These neurotrophic proteins have been assessed for their ability to protect neuronal cells in the eye from apoptotic cell death. Intraocular injections of recombinant growth factor proteins, such as CNTF, bFGF and BDNF exert a neuroprotective effect by promoting the survival of photoreceptor cells in the degenerating retina [124,125]. However, the results from these early studies in the eye were varied; some of the neurotrophic factors tested only promoted photoreceptor survival in certain models of RP, whereas some models showed no increased survival of photoreceptors after injection of any of the factors [125]. Cayouette *et al.* showed that a neurotrophic factor originally isolated from RPE cells, pigment epithelium-derived factor (PEDF), protects to some extent against the apoptosis seen in the *rd* mouse and the *Prph2*$^{Rd2/Rd2}$ mouse [126]. Following intravitreal delivery of recombinant PEDF protein (rPEDF), a transient reduction in apoptotic cell loss was shown in both models evaluated; three days after treatment significantly more photoreceptors remained in rPEDF-injected eyes when compared to β-Gal treated

controls, but this difference was no longer apparent six days later. Nonetheless, these were the first studies to show any efficacy of pharmacological intervention in models of inherited retinal dystrophies. An effective anti-apoptotic treatment regimen has the potential advantage that it could be applied to a wide spectrum of retinal degenerations with unrelated causative mutations, by enhancing photoreceptor survival. However, delivering the neurotrophic factors as recombinant protein is not ideal; without repeated administrations, the relatively short half-life of the proteins means that any functional improvement is short-lived. Moreover, it seems that neurotrophic factor administration alone may not suffice in models of disease where the absence of a gene product commits photoreceptors to die unless this underlying defect is also corrected. Neuroprotection via delivery of genes encoding these neurotrophic factors, however, would allow for long-term therapeutic effects of the likes of BDNF and GDNF, and this has been made possible by advances in viral gene therapy.

1.10.2 Cellular therapy for retinal degeneration

As pharmacological therapy is largely ineffective for most inherited retinal degenerations, novel treatment strategies have been developed that address the mutations underlying disease, or that attempt to stably deliver factors that enhance photoreceptor survival in mutation-independent approaches. Although the studies presented here focus on gene therapy using viral delivery of therapeutic nucleic acids, some forms of inherited retinal dystrophy may also be amenable to cellular therapy (**Chapter 1.12**).

Cellular therapy is a term that describes three broad therapeutic approaches; replacing the diseased cell type with healthy tissue; treatment with an ectopic cell type that provides trophic support for degenerating photoreceptors; or treatment with a cell type engineered to secrete a growth factor that enhances photoreceptor survival. An example of the first strategy would be the transplantation of healthy RPE cells to treat disease caused by RPE-specific mutations. This has been shown to be effective in models of RP, and in patients with AMD. In particular, the photoreceptor

degeneration in the RCS rat may be slowed to a certain extent by transplantation of immortalised human RPE cells (ARPE19), with an improvement in behavioural function correlating to some preservation of photoreceptors [127,128]. In addition, engraftment of a healthy region of RPE underneath a diseased macula improves visual function in some AMD patients [129], although other studies carried out indicate that the occurrence of proliferative vitroretinopathy [130] and/or secondary geographic atrophy [131] may hinder functional recovery following such procedures. In addition, these and other studies using RPE translocation require autologous cell transfer, or require the recipients to be immuno-suppressed in order to avoid graft rejection.

An ideal cellular therapy would be the use of stem, progenitor, or precursor cells to regenerate tissue that is damaged in disease. With the discovery of neural stem cells in mature mammalian eyes [132], the possibility of transplanting progenitor cells into the eye to repopulate the degenerating retina arose. Early work using GFP-positive donor cells transplanted into degenerating recipient retinae showed limited integration of transplanted cells into the outer nuclear layer, with most cells that survived the graft doing so in the plexiform layers or the inner nuclear layer of the retina [133]. Enhanced survival of host photoreceptors and a modest improvement in visual function was reported. However, the transplanted cells used in this study were isolated from neonatal mice and included many different cell types at various stages of development. It is therefore likely that the improvements seen resulted from engrafted cells secreting soluble trophic factors as opposed to integrated and differentiated donor cells replacing degenerating photoreceptors. Subsequent studies have attempted to restore structure and function to degenerating retinae using transplantation of various populations of retinal progenitor cells. To date many such studies have attempted to use actively dividing retinal progenitor cells transplanted into degenerating recipient eyes. This was based on these transplanted cells receiving the appropriate developmental cues from the environment into which they integrate and developing into functional rods and cones. However, it has now been shown that although precursor cells from an immature donor eye can integrate and develop into functional rods, these donor cells have to be post-mitotic cells committed to a photoreceptor fate for this integration to be successful. This was determined using donor cells from mice in which a GFP gene is driven by the *Nrl* promoter, which is the earliest rod-specific transcription factor expressed in post-mitotic rod precursors

[26]. Using cells from post-natal *Nrl.GFP* mice, it has been shown that only non-dividing, post-mitotic (and hence GFP-positive) precursor cells are able to integrate into an adult recipient retina, although actively-dividing cells do survive engraftment in the sub-retinal space [134]. In many studies of this nature, the precursor cells are generally taken from the ciliary margin, a region of the anterior retina near the ciliary body. The presence of these 'stem-like' cells in the adult has been described as a 'niche' for cells that could potentially repopulate damaged areas of the host retina; however, in mammals although these precursor cells have the ability to differentiate when removed from their native environment, they do not appear to do so *in situ* during the course of disease. This, together with the results described by Maclaren and Pearson *et al.* [134], suggests that although the adult retina can host engrafted retinal precursors, early and undifferentiated stem/precursors are unable to integrate and subsequently develop into mature photoreceptors, presumably as the adult retina lacks the requisite developmental cues in the form of transcription factors and other signalling molecules [134]. Another cellular therapy approach is to use cells such as Schwann cells to provide trophic support to degenerating retinae. Initially Schwann cells were shown to aid regeneration of CNS lesions in rats [135]; subsequently Schwann cell have been used to aid neuronal regeneration in a contusion model of spinal chord injury in rats [136] and to promote retinal ganglion cell survival following optic nerve transection [137,138]. It has been shown that the ability of Schwann cells to promote axonal regeneration and neuronal cell survival depends on the secretion of neurotrophic factors such as NGF [136]; it follows that Schwann cells, or indeed many other cell types, engineered to over-express neurotrophic genes could exert a similar neuroprotective effect. This is supported by evidence from studies in which Schwann cells transduced *ex vivo* to produce brain-derived neurotrophic factor (BDNF) promote axonal regeneration across a spinal chord transaction [139]. In the eye, Schwann cells transfected *ex vivo* with plasmids encoding BDNF and glial cell line-derived neurotrophic factor (GDNF) prolong photoreceptor survival and provide some modest behavioural improvement in the RCS rat, although untransduced Schwann cells also provide some transient improvements [140]. Cellular implants that are engineered to secrete CNTF have been used to treat patients of retinitis pigmentosa, and this will be discussed in further detail in the context of AAV-mediated *CNTF* delivery in a rodent model of RP (**Chapter 5**). Aside from using cellular therapies for inherited retinal degenerations,

the use of gene therapy strategies holds much promise and it is the aim of these studies to develop existing approaches to ocular gene therapy.

1.10.3 Gene therapy for retinal degeneration

Gene therapy refers to a range of molecular techniques designed as rational strategies to treat disease by delivery of nucleic acids. There are several approaches that can be used to deliver nucleic acids to a target organ using a vector. The vector used can be viral or non-viral, with greater efficacy associated with the former whilst the latter offers greater safety. For most ocular gene therapy applications, viral vectors are preferred; this is because highly efficient transduction of cells is required to mediate functional benefit, and cell-specific expression can often be critical. Which particular viral vector is used depends on the cell type to be treated.

The eye is regarded as a good target organ for gene therapy. Primarily this is due to the ease of access, both for delivery of gene therapy agents and with regards to non-invasive follow up. Administering therapeutic agents through intraocular or sub-retinal injections is not disruptive beyond the level of procedures carried out routinely in ophthalmic surgery. Ophthalmoscopy or electroretinography, used to assess post-treatment retinal function, are non-invasive and easily standardised. Furthermore, the eye contains cell types from different embryological origins and with diverse functions, and hence can be a valuable resource for testing gene delivery to several tissue types. Moreover, the blood-retina barrier allows the expression of ectopic genes to be targeted to specific cell types and systemic spread of virus and transgene can be limited [141]. The blood-retina barrier, composed of tight junctions between RPE cells and between corneal epithelial cells, also prevents the entry of large pathogens into the eye through the blood supply. Additionally, the eye carries out many more immuno-modulatory functions in order to avoid inappropriate inflammatory responses to antigens (reviewed by Streilein, ref. number [142]), which in itself can cause much damage to the cells in the eye; these include down-regulation of MHC Class I expression in corneal antigen-presenting cells (APCs); cytokines present in the aqueous humour that suppress both T cell differentiation and their secretion of pro-inflammatory signalling molecules; the inhibition of T cells and complement by cell-

surface proteins expressed constitutively by corneal and retinal pigment epithelium. Together these measures serve to modulate the immune response to pathogens (and, potentially, gene therapy vectors) that, if left uncontrolled, would damage the neurosensory retina. These characteristics of the mammalian eye make it an attractive target organ for gene therapy, and to date a number of vectors have been used to deliver genes to the eye with varying degrees of success.

1.11 Gene delivery vectors

1.11.1 Non-viral gene delivery

At its most basic, gene delivery to target tissues can be achieved by electroporating or even injecting cells *in situ* with naked plasmid DNA, as reviewed by Prud'homme *et al* [143]; the route of administration of plasmid DNA prior to electroporation plays an important role in determining which cell types are transduced, which will also be discussed in the context of viral gene delivery below. Short-lived (less than two weeks) localised reporter gene expression has been reported using direct injection into the corneal stroma [144]. Following anterior chamber injection of plasmid DNA followed by electroporation, reporter gene expression in corneal endothelium is maximal just three days post-treatment, and is undetectable three weeks after injection [145]. Sub-retinal injection of plasmid DNA followed by electroporation results in transient RPE-specific transgene expression, although this expression reaches a peak three days post-treatment and is undetectable 21 days post-treatment [146]. The transient nature of reporter gene expression using this technique could be because host enzymes such as DNAse break down naked DNA, leading to poor transduction rates. Neonatal gene transfer using electroporation appears more efficient, with up to 80% of rod cells transduced by a reporter gene expression cassette following treatment at post-natal day 0 [147]. Rather than electroporating naked plasmids, DNA can be delivered to cells in liposomes, an approach that has been used in tissues such as muscle [148,149]. In the eye, lipofection can lead to transient reporter gene expression, but lasts no longer than 21 days. Cationic polymers

have been assessed for their ability to deliver DNA to target cells; advances are being made using this approach, with improvements in transduction efficiency a priority [150]. Because the DNA remains episomal, in dividing cell the transgene is lost during cell division, which also reduces the longevity of expression following non-viral gene delivery; nonetheless as many cell types in the eye are post-mitotic, therapeutic levels of transgene expression may be achieved using these vectors. Novel transfection agents that incorporate ligands for specific cell-surface receptors have also been developed, allowing receptor-mediated endocytosis of cationic polymer-DNA complexes to increase transfection efficiency and specificity of cell types transduced; for instance, by incorporating the protein transferring into PEI/DNA complexes, highly specific gene transfer to hepatic tumours can be achieved, although it is not clear whether this expression would persist beyond the one week time-point assessed in this study [151]. Despite these advances, *in vivo* gene delivery using these non-viral techniques remains of low efficiency and are of most use when transient gene expression is sufficient to mediate a treatment effect [152]. This is possibly because the DNA introduced into host cells using electroporation and/or lipofection remains episomal. Recently Chalberg et al. have shown that electroporation leads to persistent reporter gene expression (at least 4.5 months) in rat RPE following sub-retinal injection of naked plasmid DNA, if co-injected with a plasmid encoding the *integrase* gene from the phiC31 bacteriophage [129]. Essentially these latest strategies used in developing non-viral gene therapy vectors have attempted to mimic mechanisms used by viruses to incorporate DNA into host cells and provide long-term, stable gene expression.

1.11.2 Viral gene delivery vectors

Viruses are naturally capable of delivering nucleic acids to the nucleus of their target cells, and viruses engineered to deliver therapeutic transgenes have been extensively studied in cell culture and animal models of disease, and to some extent in humans. The choice of which viral vector is used can be crucial to the success or otherwise of a gene therapy strategy; viruses suited for a particular cell type or a particular disorder may not be applicable to other contexts. Discussed below are the

candidate viral vectors that have been considered for use in gene therapy applications in the eye as well as in other systems.

Herpes Simplex virus (HSV)-based vectors

HSV is widely used in gene transfer experiments in organs other than the eye. A large double stranded DNA virus that naturally infects neuronal cell types, gene therapy strategies for Parkinson's disease [153] and Alzheimer's disease [154] have been based on this virus. A major advantage of using HSV to deliver therapeutic genes is the large coding capacity of the HSV genome; by replacing non-essential elements of the wild-type genome, viruses can package up to 30 kb of transgenes within multi-cistronic expression cassettes. HSV is able to establish latent infection by persisting as episomal DNA as part of its life cycle. Once this latency is established, the only viral transcripts that are produced are the latency-associated transcripts, or LATs [155]. The expression of these transcripts, which as of yet have unknown function, is driven by the LAT promoters; active during the latent phase of the HSV life cycle, these promoters can be used to drive stable transgene expression over long periods of time – in neuronal tissue stable transgene expression lasting at least six months has been demonstrated [156]. There are three ways in which recombinant HSV particles can be used as gene therapy vectors, which are applicable in different disease paradigms. Firstly, HSV can be engineered to harbour mutations in viral genes that are essential for nucleic acid synthesis, which renders the virus replication-deficient in non-dividing cell types. These mutations can be in the HSV thymidine kinase gene, and the ribonucleotide reductase gene [157], or in a gene that shuts off host-cell protein synthesis (IPC34.5) [158]. These viruses are then able to replicate in cells that have up-regulated host functions that complement those of the mutated viral gene, such as malignant tumour cells (that have re-entered the cell cycle and are in G1/S phase) [159]. Once these mutant HSV virions replicate in tumour cells, they lyse the host cells and have been shown to be well tolerated and to have anti-tumour efficacy in patients that have high grade glioma [160]. Hence HSV that replicates only in tumour cells constitutes one form of oncolytic viral gene therapy, whereby viruses are used to lyse malignant tissue; recombinant adenoviruses can also be used as oncolytic agents (see below). As gene delivery to neuronal cells such as photoreceptors could be used as a therapeutic strategy for treating RP, HSV would be

a candidate vector for delivery of therapeutic genes to the eye. Following intravitreal injection of recombinant HSV, 25% of retinal ganglion cells are transduced, but transgene expression lasts for less than two weeks; RPE expression also seen for two weeks following intravitreal HSV injection, but photoreceptors are not transduced using any route of administration [161]. This is likely to be a function of the large size of HSV virions (around 200nm), preventing virions from penetrating the inter-photoreceptor matrix and entering photoreceptors cells. Coupled with HSV's high natural immunogenicity and the difficulty of making high-titre, pure viral stock makes recombinant HSV of limited use for ocular gene transfer. A novel HSV-based gene delivery system may be of some utility in the treatment of acute ocular conditions, and is based on amplicons that can contain transgene expression cassettes of up to 150 kb. HSV amplicons are essentially plasmids which contain the HSV *ori* sequence (origin of replication) and the HSV *pac* (packaging) sequence and an expression cassette carrying the gene of interest. To produce an amplicon-based recombinant HSV stock, the amplicon plasmid is transfected into a permissive cell line, and is either packaged into mature HSV particles using replication-defective HSV in which the packaging signal has been deleted, or by providing the necessary HSV capsid proteins *in trans* using a cosmid or BAC vector (for review see ref. [162]). Rapid RPE-specific transgene expression was reported following administration of an HSV amplicon-based vector following sub-retinal delivery [163], which was nonetheless transient. In around 10% of cells this may have been because the promoter activity from the episomal transgene expression cassette was shut off. The majority of cells appeared to have lost their episomal amplicon, possibly through an immune response to the vector preparation. Therefore where acute transgene delivery would be able to mediate a therapeutic effect, these amplicon vectors may play a role.

Adenoviral vectors (Ad)

Adenovirus (Ad) was the vector of choice for many early *in vivo* gene transfer experiments, and has shown to mediate effective therapeutic gene expression in several species [164]. Clinical trials using Ad-based vectors have also been carried out, with diseases such as cystic fibrosis [165] and cancer [166] being treated. A major advantage of using Ad-based vectors is that a relatively large amount of DNA can be incorporated into their genome; transgenes of up to 48 kb can be delivered to

host cells using recombinant adenovirus, whereas the lower coding capacity of other vector systems can be a limiting factor for gene delivery. Much work has been carried out to develop safer and more efficient adenoviral vectors, with progressively fewer and fewer viral genes being included within the mature recombinant virion [167]. The first Ad-based vectors were made replication-deficient by deletion of the E1 region of the adenoviral genome, which contains genes involved in regulation of early phase of the infectious cycle. 'Second-generation' Ad vectors are further deleted in the E4 or E2 regions, preventing transcriptional control of viral gene expression and viral genome replication respectively. In the eye, as with other viral and non-viral gene delivery vectors, the route of administration determines which cell types are transduced by adenoviral vectors. Following anterior chamber injection of an adenoviral vector, reporter gene expression is seen in the corneal endothelium, iris epithelium, and trabecular meshwork, whereas sub-conjunctival injection results in conjunctival fibroblasts being transduced and sub-retinal injection results in exclusively the RPE being transduced [168]. However, the utility of first and second generation adenoviral vectors for ocular gene delivery is limited due to the aggressive immune response initiated by adenovirus in the eye; T-cell mediated immune responses lead to abolition of transgene expression as early as three weeks after administration to the retina, whereas mice lacking a T-cell repertoire maintain high levels of transgene expression for at least four months [168]. In retinal gene therapy applications where long-term, therapeutic gene expression is required, therefore, Ad-based vectors are extremely limited in their utility. The clinical use of this vector system has been questioned following the death of a patient during a phase I trial for adenoviral vectors in ornithne trascarbamylase deficiency (OCD) [169]. In retrospect, the phase I clinical trial may have been poorly planned due to a relative paucity of *in vivo* studies in animal models of OCD. Given that some of the early studies involving adenoviral vectors in models of OCD had been carried out in neonatal mice, which have no cellular immune response, it is possible that the investigators underestimated the potential adverse effect this adenoviral vector could have following high-dose hepatic administration. It was an aggressive systemic immune response to a high dose of adenoviral vector that caused the lethal complications seen in this clinical trial Despite a small study in non-human primates demonstrating that this vector is well-tolerated at low-to medium level doses (up to $6x10^{11}$ particles per kg) [169], this same dose range resulted in severe and lethal inflammatory side effects in the phase I trial.

This lead to the suspension of all gene therapy trials by the United States Food and Drug Administration (USFDA), and to a more cautious approach to adenoviral gene therapy. Despite this major setback, clinical trials for gene therapy applications are now being approved for a range of diseases and vectors [170], and in China an adenoviral construct encoding *p53* has recently been approved as a licensed treatment for head and neck squamous cell carcinoma (HNSCC) [171]. Gendicine, an Ad-5 serotype vector, has been used to treat in excess of 2600 HNSCC patients. Indeed, the development of safer, less immunogenic helper-dependent adenoviral vectors [172] have allowed gene therapy for OCD to be investigated once again, with longer-term disease correction and less of a deleterious immune response in mice [173].

In the eye, adenoviral vectors have been used for a Phase I clinical trial for the choroidal neovascularisation (CNV) associated with age-related macular degeneration (AMD). This open-label trial demonstrated that adenovirus-mediated *PEDF* expression is well tolerated in patients with severe neovascular AMD, although very low doses of adenovirus were used [174]; studies in which monkeys were injected with increasing doses of the Ad.PEDF vector had identified $1x10^9$ particles as being the maximum tolerated dose, beyond which an adverse immune reaction to the vector was noted [175] – in the clinical trial doses between $1x10^6$ and $1x10^9$ were used. Data relating to efficacy of this treatment in ameliorating vision needs to be interpreted with caution due to the small scale of the trial, in which 28 patients were treated. This is particularly applicable to this trial, as the natural history of AMD patients' visual acuity is such that some month-on-month improvement in outcome measure can be expected without intervention, and also because visual acuity may not always be the most appropriate way of assessing functional vision. Despite the potentially beneficial effects of PEDF in protecting retinal neurones from apoptosis, delivery using Ad-based vectors appears to be counter-productive due to the high likelihood of inflammatory responses to the vector itself. It may well be that gutted or helper-dependent Ad-based vectors are appropriate when transient gene expression allows long-term disease correction; in order to mediate effective gene therapy in most ocular diseases, however, vectors need to drive stable, long-term gene expression in the absence of immune responses and other deleterious effects.

'Third-generation' or 'gutted' Ad vectors have recently been developed, and are deleted for all viral genes. They therefore offer two major advantages; the host immune response seen with earlier Ad vectors is greatly diminished, and the gutted vectors also have a capacity to deliver larger expression cassettes. This increased cloning capacity is of particular interest, especially with respect to multi-cistronic approaches to gene therapy. The use of gutted Ad vectors results in longer-term transgene expression in mice, and is well tolerated because little immune response is provoked; in a model of muscular dystrophy, intramuscular delivery of a gutted adenovirus encoding murine *dystrophin* gene results in efficient expression of dystrophin protein in the absence of vector- or transgene-mediated immune response [176]. This gene replacement therapy continues to rescue 50% of muscle fibres in the dystrophin-deficient (*mdx*) mouse one year post-injection from contraction-mediated injury [177]. In the eye, Ad-mediated transgene product persists in the form of mRNA for at least 18 weeks post-treatment and protein for up to five months in *rd1* mice treated with sub-retinal delivery of Ad.βPDE [178]. Although the latest Ad vectors that are deleted for all viral genes are promising in terms of their ability to drive high levels of transgene expression without eliciting a host immune response, there remain technical barriers to large-scale, clinical grade adenovirus production.

Retroviral vectors

A third group of viral gene transfer vectors based on retroviruses have been developed that mediate long-term, highly efficient transgene expression. Retroviruses are RNA-based viruses that integrate their reverse-transcribed proviral DNA into host cell chromosomes, and are classified into seven distinct genera according to their sequence similarity (**Table 1.2a**); these genera share common features, such as a ~10 kb RNA genome that encodes *gag*, *pol* and *env* genes (see **Table 1.2b** and **Fig. 1.14** for details), and their overall genomic organisation whereby the integrated provirus has 3'- and 5'-long terminal repeat (LTR) sequences flanking these genes.

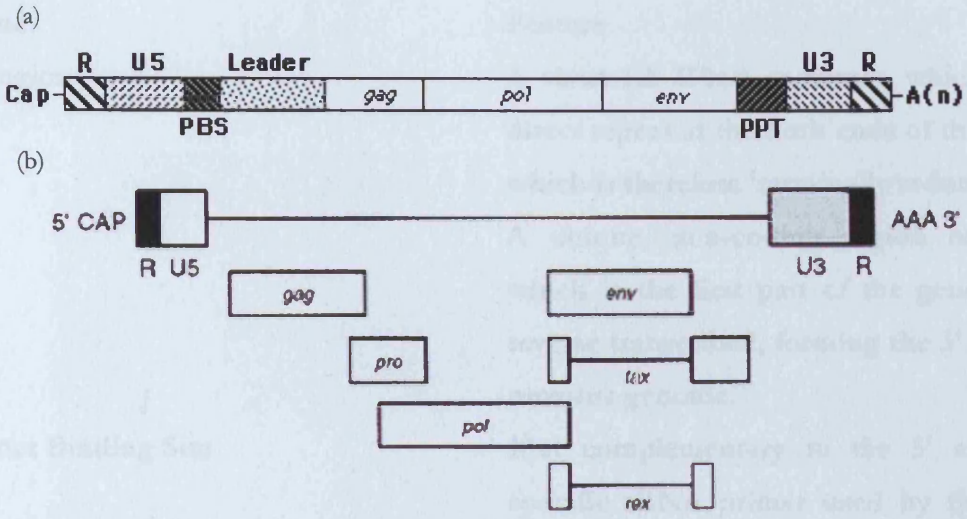


Fig. 1.14: (a); Illustration of major features common to retroviral genomes. **(b);** genes other than *gag, pol* and *env* found in lentiviral genomes. These are all deleted in retro- and lentiviral vectors, rendering them replication-deficient. Taken from diagrams available at http://www.virology.net and from ref. number [179]

Genus:	Type Species:
Avian type C retroviruses	avian leukosis virus (ALV)
BLV-HTLV retroviruses	bovine leukaemia virus (BLV)
Lentivirus	human immunodeficiency virus (HIV-1)
Mammalian type B retroviruses	mouse mammary tumor virus (MMTV)
Mammalian type C retroviruses	murine leukaemia virus (MLV)
Spumavirus	human spumavirus (HSRV)
Type D retroviruses	Mason-Pfizer monkey virus (MPMV)

Table 1.2a: Classification of retroviruses into genera according to sequence similarity. Type species, right column; example of a retrovirus belonging to the genus shown in left column. Table available at http://www.virology.net

Name	Feature
R Region	A short (18-250nt) sequence which forms a direct repeat at the both ends of the genome, which is therefore 'terminally redundant'.
U5	A unique, non-coding region of 75-250nt which is the first part of the genome to be reverse transcribed, forming the 3' end of the provirus genome.
Primer Binding Site	18nt complementary to the 3' end of the specific tRNA primer used by the virus to begin reverse transcription.
Env	Encodes a 160 kd precursor protein, which is cleaved posttranscriptionally into two envelope glycoproteins. The first env protein is gp120, a highly charged glycoprotein that binds to cell-specific viral receptors (e.g., CD4+ T cell receptor in the case of HIV) and ancillary coreceptors (e.g., chemokine receptors for HIV-1). There are five highly variable domains of the env gene - V1 through V5 – which are responsible for viral evasion from host immune response. The second env protein, gp41, is a hydrophobic transmembrane glycoprotein that anchors gp120 to the virus envelope.
Central Polypurine Tract (cPPT)	~10 A/G residues responsible for initiating (+) strand synthesis during reverse transcription.
U3	A unique non-coding region of 200-1,200nt which forms the 5' end of the provirus after reverse transcription; contains the promoter elements responsible for transcription of the provirus.
Gag	Gene encoding the MA, CA, and NC

	proteins, which are group-specific structural antigens
pro	**Gene encoding the viral protease. For most retroviruses, the *pro* ORF is -1 with respect to the *gag* ORF and a Gag-Pro polyprotein is expressed by a -1 frameshift during translation.**
pol	**Gene encoding the viral reverse transcriptase and integrase proteins. In most cases (except spumaviruses), the gene product is synthesized as a 180-kD Gag-Pro-Pol precursor polyprotein. In mammalian type-C, HTLV/BLV, lentiviruses, WDSV, and Gypsy, the *pol* ORF is in-frame and contiguous with the *pro* ORF producing equimolar amounts of Pro and Pol proteins.**

Table 1.2b: Features of retroviral genomes common to all viruses in this genus.
Adapted from http://www.virology.net

Vectors based on type-C retroviruses such as Moloney murine leukaemia virus (MoMLV) have shown considerable promise in gene therapy [180]. Vectors based on MoMLV, a 100 nm single-stranded RNA virus with a genome of 8.3 kb, are the most commonly used retroviral vectors in animal trials of gene transfer. To generate recombinant retroviruses the gene of interest is normally cloned into a plasmid containing 3'- and 5' long terminal repeat (LTR) sequences and the 350 bp ψ sequence required to package this recombinant genome; the *gag* and *pol* genes required to generate virus capsid are provided *in trans*, in helper plasmids that are ψ-negative. Packaging cell lines stably transfected with plasmids harbouring mutations in both the ψ sequence required for packaging and complementary mutations in the *gag* and *pol* genes are now used; this avoids the possible formation of replication competent retrovirus during virus production, by ensuring that in the rare occasions where the ψ-negative helper plasmid is packaged into retroviral virions, they are not replication competent [181]. Sustained therapeutic efficacy has been demonstrated using an MoMLV vector in human clinical trials of gene therapy for X-linked severe

combined immune deficiency (X-SCID), an immune disorder caused by a mutation in the gene encoding the γ-chain common to cytokine receptors (*IL2RG*) [182]. X-SCID patients fail to develop NK and T-cells due to a block in the differentiation of T-cells in early haematopoiesis. The result is a severely compromised cellular immune system that is lethal without allogenic bone marrow transplant [183]. The first clinical trial to use gene delivery using MoMLV-based vectors demonstrated effective disease correction in five X-SCID patients. They had received autologous bone marrow grafts that had been transduced *ex vivo* with an MoMLV vector expressing the γ-chain gene, which conferred a selective advantage upon the transduced cells when grafted into the host patients. This allowed effective repopulation of T-cells, and allowed the restoration of a normal T- and B-cell repertoire that lead to these previously immuno-compromised patients having a much improved quality of life [184]. Indeed, a longer-term follow up of these patients revealed no significant adverse events, and a second trial using a similar study design was undertaken using a separate patient cohort. This second trial also showed sustained disease correction in X-SCID patients after *ex vivo* retrovirus-mediated gene transfer, and six years after treatment no serious side effects have been reported. However, as with Ad-based gene transfer vectors, efficacy of retroviral vectors in the clinic has been accompanied by serious adverse events in the first trial described above. Three of the twenty patients treated to date developed clonal expansion of transduced T-cells, resulting in acute lymphoproliferative disease following treatment with a MoMLV vector [185]. The neoplastic transformation in these patients was ascribed to vector-mediated insertional mutagenesis, due to the integration of the vector provirus in the promoter region upstream of the *LMO2* gene [185]. Although the patients concerned were treated with chemotherapeutic agents, two have this was viewed as a severe adverse event caused by aberrant overexpression of an oncogene directed by proviral integration. As such, the need to develop vectors that carried less risk of insertional mutagenesis was highlighted; despite the long-term reconstitution of a normal repertoire of T-cells, the potential for deleterious effects caused by gene transfer vectors is of major concern. One study in mice lacking the γ-chain receptor (*IL2RG*$^{-/-}$ mice) seems to suggest that the therapeutic transgene itself is oncogenic. They claim that because *IL2RG*$^{-/-}$ mice only develop lymphoma after *ex vivo* transduction of bone marrow with an MoMLV vector encoding the *IL2RG* gene, but not a vector encoding a GFP reporter gene, that the *IL2RG* gene is what causes transformation of T-cells and not insertional mutagenesis

per se [186]. However, their data does not support this conclusion. The authors did not treat *IL2RG*$^{-/-}$ mice with wild-type bone marrow that had been transduced with MoMLV expressing the *IL2RG* gene and check for lymphoma formation, nor did they treat wild-type mice with a vector encoding a reporter gene. Therefore possibility that insertional mutagenesis *is* the principle cause of lymphoma formation, but only in the context of cells that are able to proliferate and expand (i.e. in the presence of the γ-chain receptor), cannot be excluded. Two further severe, early-onset immune disorders have been successfully treated with retroviral vector-mediated gene therapy, the adenosine deaminase deficient form of severe combined immunodeficiency (ADA-SCID) and chronic granulomatous disease (CGD). In the case of ADA-SCID, although patients may benefit from delivery of the adenosine deaminase enzyme itself [187] or from HLA-matched bone-marrow transplants [188], in many cases neither treatment is applicable. The first clinical trials of ADA-SCID involved a retroviral vector encoding the *ADA* gene, and showed that long-term reconstitution of immune repertoire in treated patients [189,190]. A more recent report of a patient previously refractory to enzyme replacement therapy benefiting from retrovirus-mediated gene replacement therapy reinforces the efficacy of this approach for ADA-SCID [191]. Gene delivery to bone-marrow in patients with chronic granulomatous disease has also been studied, with adeno-associated virus initially being used as a potential therapeutic agent [192,193]. Clinical trials of gene therapy for CGD, however, have used retroviral vectors encoding components of the NADPH-oxidase multi-enzyme complex (which is deficient in CGD patients), and have shown that patients' anti-microbial capability is significantly improved following such gene replacement therapy [194,195]. In the case of the latter study, retroviral insertion was shown to up-regulate expression of three genes that drove clonal expansion of the gene-corrected granulocytes. This unexpected augmentation of the treatment effect of gene replacement is in stark contrast to the findings in the X-SCID trial, where viral insertion lead to leukaemogenesis. This is likely due to the fact that retroviral expression of *gp91*^{phox} confers no selective advantage to transduced cells, such that even in the event of insertional activation of growth-promoting genes (as is seen in the CGD trial), no abnormal tumourigenic cell division is seen. In the case of X-SCID gene therapy, however, cells transduced to over-express the *IL2RG* gene (and hence have a selective expansion advantage over non-transduced cells) can be seen as being primed, or sensitised, to the oncogenic insertional activation of *LMO2*. Thus

leukaemogenesis following gene therapy of haematopoietic cells can be seen as a multi-step process, in which insertional activation of oncogenes is only truly oncogenic in cells possessing a selective advantage by nature of the therapy itself. The continued concern over insertional mutagenesis causing inappropriate activation of oncogenes is being addressed by the development of vectors that do not integrate their proviral genomes into host DNA, as described below. Vectors based on retroviruses are not appropriate for diseases of the retina, as they cannot transduce non-dividing cell types; for efficient transduction of cells in the retina, vectors based on a sub-category of retroviruses are used.

Lentiviral vectors

Vectors based on a sub-group of retroviruses, the lentiviruses, are currently being used in gene therapy and provide greater promise for ocular applications. Whilst lentiviruses are similar to retroviruses in that they use RNA as their genomic nucleic acid, there are many features that distinguish lentiviruses from retroviruses. Firstly, lentiviruses are diploid viruses, meaning they carry two copies of positive-sense RNA within each mature virion. In addition, lentiviral genomes encode additional genes (such as *rev, tat, vif* and *vip*, see **Fig. 1.14**) that code for proteins involved in regulation of proviral gene expression and are called 'accessory proteins'. Whereas retroviruses only transduce dividing cell types, thereby preventing the use of vectors based on MoMLV being used to treat the terminally differentiated cell types in the eye, lentiviruses mediate transgene expression in quiescent or terminally differentiated cells as well [196]. Another advantage of lentiviral vectors is that self-

inactivating vectors (see below) that are deleted for all viral genes can be made. This allows a completely replication-deficient virus to be used, providing a safe way of preventing lytic infections of host cells. In addition, recent developments in lentiviral production methods allow the prevention of previously encountered systemic immune responses; producing recombinant lentivirus using serum-free medium, and purifying the vector by sucrose gradient ultracentrifugation, abrogates the immune response seen following injection into mouse brain, demonstrating that the immune response previously seen is due to contaminating immunogenic components in the viral preparation rather than the lentiviral particles *per se* [197].

The surface glycoproteins on lentiviral envelopes determine their tropism towards given cell types; the natural tropism of wild-type lentiviruses such as human immunodeficiency virus (HIV) is restricted to $CD4^+$ T-lymphocytes, and by altering the expression of the glycoproteins on the viral envelope, lentiviral vectors can be engineered to infect a broader range of cell types. This is known as pseudotyping, and results in lentiviruses expressing envelope proteins from other viruses on their surface; striatal and hippocampal neurons [196], muscle , liver [198] and RPE [199] can be transduced by altering lentiviral tropism in this way. Pseudotyping vectors based on equine immuno-deficiency virus (EIAV) alters the transduction profile of this non-primate lentivirus. EIAV vectors pseudotyped with the varicella simplex virus surface protein (VSV-G protein) result in efficient transduction of corneal endothelium and some expression in the trabecular meshwork following anterior chamber injection; when pseudotyped with the rabies-G protein, however, there is no transduction of these cell types, but neuronal cells in the cornea and iris appear transduced. EIAV.VSV-G vectors do give some expression in photoreceptors, although this occurs with greatly reduced transduction efficiency than in RPE cells, which show very high levels of reporter gene expression [200]. Thus far, RPE cells appear to be the only cell type transduced with high enough efficiency for therapeutic purposes in the context of retinal dystrophies; however, this does not preclude the use of lentiviral vectors in disease paradigms in which photoreceptors die. This can be achieved by direct treatment of the RPE, whereby an RPE-specific gene that is mutated in a model of retinal degeneration is delivered by a lentivirus, or by transducing the RPE with a lentivirus encoding a secreted growth factor to provide neuroprotection to dying photoreceptors; both possibilities will be discussed below.

Another feature of the latest lentiviral vectors prevents them from activating inappropriate transcription of genes downstream of the integration site. As discussed above for retroviruses, lentiviruses reverse transcribe their diploid RNA genome, and the reverse-transcribed cDNA is integrates into the host genome essentially at random using the viral integrase enzyme. The genomic RNA contains promoter and enhancer elements in the U3 region of the 3' Long Terminal Repeat (LTR) sequence, which are only transcriptionally active when transferred to the 5' LTR during integration of the provirus. Thus the latent provirus has transcriptional activity in its 5' LTR which can trigger transcription of genes downstream of the integration site that otherwise would be silent. This is undesirable in the case of most gene therapy applications, as demonstrated in the X-SCID trial, and so late-generation retro- and lenti- viral vectors that carry deletion mutations in their 3' LTR regions circumvent this potential risk by preventing promoter activity from this modified LTR. Both retroviral [201] and lentiviral [201] vectors modified in this manner are referred to as self-inactivating, as deletions in the 3' viral LTR result in the 5' *pro*viral LTR promoter activity being abolished. Another strategy for avoiding insertional mutagenesis following lentivirus-mediated gene transfer has been to develop vectors that do not integrate their proviral DNA into the host genome. Initial work using a feline immunodeficiency virus (FIV)-based vector showed that by incorporating a point mutation in the *integrase* gene that encodes a D66V amino acid substitution, *in vivo* transduction of RPE was abrogated [202]; in the absence of evidence showing that this vector was able to reverse transcribe its genome and drive any transgene expression (from non-integrated episomes), it is unclear whether this mutation was pleiotropic and hence ablated viral transgene expression altogether. However, subsequent *in vitro* data using integrase-deficient vector based on HIV-1 showed efficient transgene expression using an SV40-*ori* promoter sequence in the presence of the SV40 Large T antigen [203]. Whereas previously it was considered that lentiviral vector-mediated expression could only be achieved from integrated proviral genomes, this study provided evidence for episomal lentiviral genomes mediating stable transgene expression in the absence of integration into host DNA, albeit *in vitro* and at short-term time points *in vivo*. These vectors were rendered integrase-deficient by the introduction of a point mutation in the *integrase* gene, giving rise to a D116N amino acid substitution; expression of reporter genes using the SV40 *ori* promoter was demonstrated in permissive cell lines. Following this short-term *in vitro* study, we have shown that integrase-deficient

vectors are able to mediate stably maintained transgene expression in the RPE *in vivo* for up to nine months [204]. We also evaluated the ability of such non-pleiotropic *integrase* mutants to mediate therapeutic gene delivery in models of retinal degeneration (see **Chapter 3**). Thus the newest lentiviral vectors incorporate additional safety features that may facilitate their use in the clinic; this generation of self-inactivating, pseudotyped and now integrase-deficient lentiviral vectors are very effective in animal gene transfer trials, although their use in gene therapy for photoreceptor-specific disorders remains limited to gene correction in the RPE, and to transducing the RPE to produce neurotrophic factors that can prevent photoreceptor apoptosis. Tschernutter *et al.* demonstrated histological and electrophysiological disease correction in the RCS rat, a model in which defects in the phagocytosis of photoreceptor outer segment material leads to rod and cone cell death [205]. Lentivirus-mediated *Mertk* expression was shown to maintain outer nuclear layer thickness, improve rod function as assayed by ERG, and restore phagosomes to the RPE cells as shown by electron microscopy. This was the first example of a lentiviral vector mediating structural and functional improvement in a model of retinal degeneration. As several retinal dystrophies are caused by mutations in genes expressed in the RPE, there are many forms of RP that could potentially benefit from lentivirus-mediated gene replacement therapy. Given the role of the RPE in providing trophic support for the neurosensory retina, and the highly efficient transduction of RPE seen with lentiviral vectors, mutation-independent treatment strategies for RP may involve the use of vectors based on HIV-1. This type of treatment strategy involves administration of vectors that encode secreted growth factors and/or anti-apoptotic factors that prevent photoreceptor apoptosis; given the highly efficient transduction of RPE achieved by HIV-1-based vectors, using the RPE to produce ectopic factors of this nature may aid RP gene therapy. Currently, however, the relative difficulty of producing clinical grade lentiviral vectors, which can be produced on a large scale and to standards approved by regulatory bodies such as the Medicines and Healthcare products Regulatory Agency (MHRA) in the UK, means that alternative vector systems are being developed for use in the clinic.

Adeno-associated virus (AAV)-based vectors

Possibly the most promising viral vector for gene therapy directed towards photoreceptors is based on the Adeno-Associated Virus (AAV). A dependovirus that is naturally replication-deficient and non-pathogenic, AAV has a 4.8 kb single-stranded DNA genome that consists of two multi-cistronic genes, *rep* and *cap*, flanked by two inverted terminal repeat sequences (**Fig. 1.15**). The genome of naturally occurring, wild-type AAV is known to integrate into host chromosomal DNA in a site-specific manner; the AAV gene *rep* mediates this integration, at a locus on human chromosome 19 [206]. The integration or otherwise of recombinant AAV (rAAV)-based vectors remains to be established, although it is believed that low-efficiency integration does occur at multiple, non-specific sites [207]. Within the virion the AAV genome is encapsidated by a 20 nm icosahedral capsid, which is made of three viral proteins (VP1, 2 and 3) encoded by the *cap* gene. AAV has a wide tropism, infecting many cell types depending on the particular serotype concerned; the serotypes differ from each other in the sequence of their capsid proteins. It is the exact configuration of these surface proteins that determines the specificity with which AAV serotypes infect cells; each of the eight serotypes used to generate recombinant gene delivery vectors to date show different cell-type specific transduction profiles [208].

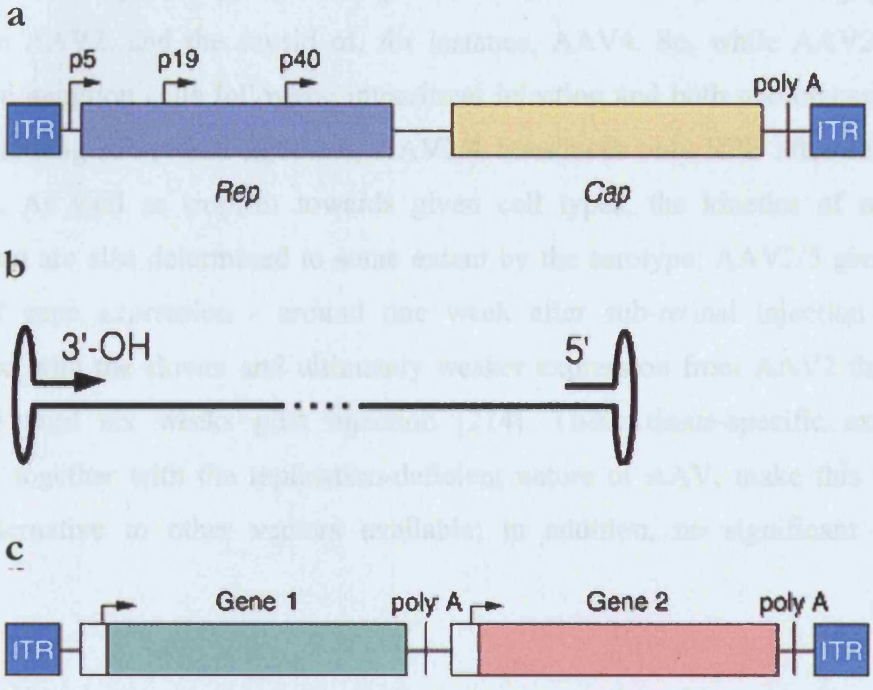


Fig. 1.15: Diagram representing adeno-associated virus genome; (a) Wild-type AAV has inverted terminal repeat (ITR) sequences at both the 5'- and 3' ends, which flank the *rep* and *cap* genes. p5 and p19 indicate the promoters that initiate the transcription of *rep* that result in the production of Rep78, Rep68, Rep52 and Rep40; p40 is the promoter that drives expression of the three *cap* proteins VP1, VP2 and VP3. (b) shows the hairpin-stem secondary structure of both ITRs, generated by Watson-Crick base-pairing within the single-stranded genome. (c) As the ITRs are the only viral sequences required for packaging into mature virions, the entire 4.8 kb genome can be replaced by expression cassettes (in this case a multi-cistronic construct) driven by ectopic promoters (black arrows).

The most widely used serotype in gene therapy applications is AAV2, as this was the first human serotype described and due to the relative ease of production of high-titre, pure virus; AAV2 binds with high affinity to heparin, making purification of virus from packaging cells relatively simple by use of heparin/agarose columns [209]. Recombinant AAV (rAAV) has been used as a gene transfer vector in various tissue types [210], and indeed has been used in clinical trials for alpha-1 antitrypsin deficiency [211] and for cystic fibrosis [212].

When delivered into the sub-retinal space, recombinant AAV2 efficiently transduces photoreceptors and RPE cells [213,214], but this tropism can be altered by using a *cap* plasmid that encodes the capsid of another serotype *in trans* during virus production. This method of pseudotyping virus produces hybrid virions, containing genomes based on AAV2, and the capsid of, for instance, AAV4. So, while AAV2 vectors transduce ganglion cells following intravitreal injection and both photoreceptors and RPE following sub-retinal injection, AAV2/4 transduces only RPE after sub-retinal delivery. As well as tropism towards given cell types, the kinetics of transgene expression are also determined to some extent by the serotype; AAV2/5 gives faster onset of gene expression - around one week after sub-retinal injection [215] - compared with the slower and ultimately weaker expression from AAV2 that is not maximal until six weeks post injection [214]. These tissue-specific expression patterns, together with the replication-deficient nature of AAV, make this vector a safer alternative to other vectors available; in addition, no significant immune

response is noted following gene delivery to the retina with recombinant AAV vectors. Hence transgene expression lasts longer after delivery with rAAV than with other vectors [214], and the safe nature of AAV allows it to mediate structural and functional improvements in several models of retinitis pigmentosa, and to be the vector of choice in clinical trials for retinal degeneration. Considering the advantages of pseudotyped AAV vectors, we decided to evaluate AAV2/5 as a gene replacement vector in three models of retinal degeneration as part of the studies presented here (**Chapter 4**).

One drawback of the rAAV system is that relatively small expression cassettes need to be used; the virions only package genome-sized lengths of DNA, meaning no more than 4.8Kbp can be cloned between the Inverted Terminal Repeat (ITR) sequences. The latest AAV vectors have an even smaller coding capacity, but have many advantages that may be of use for gene therapy applications. Self-complementary AAV (scAAV) vectors have been used *in vivo* to mediate strong transgene expression [216], and have recently been shown to be effective in gene therapy for a murine model of haemophilia B and in macaques [217]. Although an expression cassette of only 2.4 kb can be used, for many applications in the eye this may be sufficient. Initial data from our group shows faster initiation of gene expression in eyes that received sub-retinal injection of scAAV than titre- and serotype- matched single-stranded AAV (Dr. M. Rajah, personal communication). This approach may increase further the efficiency of AAV-mediated gene delivery to the retina, which using single-stranded AAV has been shown to be highly efficient and safe.

1.12 Gene therapy approaches animal models of inherited retinal degeneration

Two broad strategies have been used to 'rescue' retinal cells from degeneration – one is gene correction, which may be achieved by gene replacement or ablation of a 'poisonous' message, and the other is known as neuroprotection. In gene replacement strategies, a functional copy of the gene known to be mutated in a given

form of RP is introduced to ameliorate the pathology caused by the mutation; in order to reduce the expression of mutant genes that cause toxicity, several techniques can be used to ablate mRNA, as discussed below. Neuroprotection involves delivery of genes that do not necessarily relate to the underlying causative mutation, but that prolong photoreceptor survival. Gene therapy approaches using viral vectors have been shown to ameliorate disease in various models using both strategies, including the *Prph2*$^{Rd2/Rd2}$ mouse and RCS rat.

1.12.1 Gene correction – ablation of toxic message

Gene replacement is essentially only useful in the case of truly recessive disorders or those involving haploinsufficiency; cases where expressing a functional allele would provide sufficient protein levels to restore function. Disorders that incorporate any element of dominance, or in which mutant (dominant negative) alleles interfere with native wild-type alleles, would benefit less from gene replacement. Such disorders would require ablation of the mutant allele either by a mutation-independent approach (whereby all transcripts of the gene of interest are ablated, then replaced by a non-silenced version), or an approach where only mutant transcripts are ablated, leaving wild-type message uncleaved. Both mutation-independent and mutation-specific approaches can be mediated by the use of ribozymes or RNA interference. Ribozymes are catalytic RNA molecules that bind to specific mRNA sequences and catalyse the cleavage of the target message; naturally-occurring ribozymes are found in plant viroids, and 'hammerhead' ribozymes can be engineered to ablate specific messenger RNA sequences that are associated with disease [218]. No *in vivo* data is available at the time of writing that validates the use of ribozymes to silencing mutant transcripts in models of inherited retinal degeneration, although *in vitro* data has demonstrated that ribozymes directed against the 5' untranslated region of *rhodopsin* mRNA could cleave all *rhodopsin* transcripts, mutant or wild-type, and that a *rhodopsin* gene designed not to be cleaved by these ribozymes could replace the ablated gene product [219]. In a mutation-specific approach, a hammerhead ribozyme directed specifically against the P23H *rhodopsin* allele, known to cause retinal degeneration in mice and humans, was shown to reduce levels of P23H mutant

transcript by 40% *in vitro* [220]. *In vivo* delivery of an AAV vector encoding this ribozyme was shown to reduce mutant transcript by around 15%, reduce loss of photoreceptors by up to 45% (compared to uninjected eyes, 20% compared with non-catalytic ribozyme) and enhance average ERG b-wave by up to 100% (although the non-catalytic ribozyme does improve b-wave by around 60%, probably by binding to the mRNA as an anti-sense oligonucleotide) [221]. Indeed, ribozyme therapy against the P23H mutant rhodopsin transcript results in protection from apoptotic loss of photoreceptors up to eight months-post-injection, although improvements in retinal function are difficult to interpret due to small numbers of rats being treated [222]. Another way of ablating a dominant negative mutant transcript is to use RNA interference (RNAi); this approach exploits the site-specific cleavage of a target mRNA molecule catalysed by a double-stranded RNA, with homology to the target, that recruits proteins such as Dicer to a multi-protein complex termed RNA-induced silencing complex (RISC) (for extensive review of RNAi and the mechanisms involved, see [223]. siRNA molecules, 21-23 nucleotide molecules that initiate RISC formation and cleavage of a target message, can be delivered directly as oligonucleotide 'drugs' to mediate therapeutic benefit; this approach is being pursued to reduce levels of vascular endothelial growth factor receptor 1 (vegfr1) mRNA to treat the choroidal neovascularisation (CNV) seen in age-related macular degeneration (AMD) – following demonstration of efficacy in mice [224], a Phase I clinical trial in AMD patients has been initiated. A more common way of using RNAi, however, is to generate short-hairpin RNA (shRNA) molecules from DNA templates; this allows the delivery of a DNA template using a viral vector, and the shRNA that is transcribed from an RNA polymerase III promoter forms a catalytically active hairpin that cleaves a target message. shRNA molecules have been shown to silence more than 90% of mutant (P23H) *rhodopsin* transcript *in vitro* and in cultured retinal explants, which can then be replaced by a *rhodopsin* gene that isn't silenced by the shRNA [225,226]. Thus it is possible to ablate mutant transcripts that cause retinal degeneration, although introducing functional copies of a mutated gene, referred to here as gene replacement, has shown more promise as a strategy to treat inherited retinal disorders.

1.12.2 Gene correction – gene replacement therapy

Early attempts to rescue the *rd1* mouse using adenoviral [227] and lentiviral [228] vectors encoding *β-PDE* were largely unsuccessful due to the failure of both of these vectors to efficiently transduce photoreceptors; vectors based on Ad [229] and HIV-1 [199] are known to mediate efficient transgene expression in RPE, but not in photoreceptors, hence the paucity of rescue using these vectors for a photoreceptor-specific disorder. As AAV2 is known to transduce photoreceptors efficiently [213], it is interesting to note that only faint β-PDE immunostaining and a slight shift in *in vitro* electrophysiological response is seen in the *rd1* mouse following AAV-mediated gene replacement therapy [230]. This paucity of rescue is discussed below in the context of more successful gene therapy approaches for inherited retinal degeneration.

Several inherited retinal dystrophies are caused by mutations in genes expressed in the RPE; specifically, extensive work has been carried out on models for LCA, a severe, earl-onset form of retinitis pigmentosa (see **Chapter 1.6**). One such animal model of LCA, the *RPE65*$^{-/-}$ mouse, loses photoreceptors slowly (>50% photoreceptors survive at 4 months of age), has an undetectable scotopic ERG from birth, and a reduction in the amount of opsin present in photoreceptors [231]. *RPE65*$^{-/-}$ mice treated *in utero* with AAV2/1.CMV.RPE65 show expression of *RPE65* at least 3 months post-injection, significant improvement in retinal function as assayed by ERG, and correction of the biochemical defect caused by the absence of RPE65, as assessed by regeneration of rhodopsin following light excitation [232]. The *rd12* mouse is a naturally occurring model of LCA which is homozygous null for a C to T mutation resulting in a premature stop codon at position 44 of the murine *RPE65* gene [74]. Gene replacement therapy in *rd12* mice using AAV2/5.RPE65 reduces accumulation of lipid droplets in RPE cells, and significantly slows the loss of photoreceptors; *rd12* mice lose one-third of their outer nuclear layer by 7 months of age, AAV2/5.RPE65-treated mice retain near-normal retinal morphology with up to ten layers of photoreceptor nuclei [233]. This improved morphology correlates with dark- and light- adapted ERG responses being restored to wild-type levels, and the treatment with AAV2/5.RPE65 also restores vision-dependent behaviour to wild-type levels [233]. Lentivirus-mediated gene replacement therapy in the *rd12* mouse has been

evaluated as part of the studies presented here (see **Chapter 3**). Furthermore, several groups have demonstrated efficacy of AAV-mediated gene replacement therapy in Briard dogs with inherited retinal dystrophy, a large animal model of LCA caused by a four base-pair deletion mutation in *RPE65* [234]. AAV2.RPE65-mediated gene replacement restores RPE65 protein production, and results in an improvement in retinal function as assayed by ERG; affected Briard dogs that had virtually undetectable b-waves pre-treatment had b-wave amplitudes 3 months post-injection of around 120 μV, which is close to one-third of wild-type levels [235]. Importantly, it has been demonstrated that AAV-mediated gene replacement therapy in this large animal model of LCA results in long-term improvements in retinal morphology and function [236,237], indicating that patients with mutations in *RPE65* may also benefit from AAV-mediated gene replacement; ethical approval for a clinical trial in LCA patients with *RPE65* mutations has been granted by the appropriate regulatory bodies both in the UK and in the USA. Another form of severe, early-onset retinitis pigmentosa is caused by mutations in *Mertk*, as discussed above (**Chapter 1.6**). The RCS rat is a well-characterised rodent model for RP caused by mutations in *Mertk*, and following both AAV-mediated [238] and lentivirus-mediated [205] *Mertk* gene delivery, treated eyes show significantly higher numbers of photoreceptors (2.5-fold more) and increased ERG b-wave amplitude (up to four-fold higher) when compared to contralateral uninjected eyes, with this treatment effect being enhanced using the lentiviral vector. The amelioration in pan-retinal function as assayed by ERG is transient, however, with AAV.Mertk-mediated gene replacement resulting in a significant improvement in b-wave for 9 weeks post-injection [238], and lentiviral gene therapy improving function for up to four months [205]. Despite this, there is strong evidence to suggest that gene replacement therapy directed to the RPE represents a promising therapeutic strategy for treating RP; as demonstrated by studies involving gene replacement of both *RPE65* and *Mertk*, correcting an RPE-specific defect can result in excellent functional recovery. This may be because the photoreceptors in retinae affected by mutations in RPE-specific genes are healthy, and so restoring function to the underlying RPE allows these inherently healthy photoreceptors to function normally. Although some forms of RP may benefit from gene replacement therapy to the RPE, however, many forms are caused by mutations in gene expressed in photoreceptors; gene replacement has proven more challenging

when this is the case, despite some success in delivering therapeutic nucleic acid to photoreceptors.

Recently gene replacement studies carried out in a model of RP have shown that AAV-mediated delivery of *RPGRIP*, whose expression is driven by a novel fragment of the murine rhodopsin promoter (mOPS) results in a restoration of RPGR and opsin protein localisation to the outer segments of photoreceptors, and a significant slowing of photoreceptor loss; the ONL in AAV.mOPS.RPGRIP-treated eyes consisted of up to 3 more rows of photoreceptor nuclei when compared to untreated contralateral controls [239]. These morphological improvements were accompanied by marked electrophysiological improvements; b-waves recorded from AAV.mOPS.RPGRIP-treated mice were on average two-fold higher in treated eyes (118 μV) than in untreated eyes (66 μV) 5 months post-injection [239]. This study shows that, although it is difficult to precisely correlate the number of surviving photoreceptors to ERG amplitude, in general if surviving photoreceptors have intact components of phototransduction an increase in photoreceptor survival does translate into an improvement of function, whilst the reverse is not always the case. A model of an X-linked retinal degeneration caused by a mutation in a gene expressed in photoreceptors has also been shown to benefit from AAV-mediated gene replacement therapy. X-linked retinoschisis causes macular cyst formation and a deficiency in inner retinal response to light excitation resulting in an electronegative ERG b-wave [240]; this disease was shown to segregate with mutations in *RS1*, a gene encoding retinoschisin [241], a protein thought to be involved in cell-cell interactions between various cell types in the inner retina during development and that is expressed in photoreceptor inner segments in the adult murine retina [242]. Mice lacking *Rs1h*, the murine orthologue of *Rs1*, have disrupted photoreceptor outer and inner segments, disorganised inner nuclear layer and mislocalisation of some retinal ganglion cells to the inner plexiform layer, as well as severely reduced ERG b-wave amplitudes when compared to wild-type levels. Intravitreal delivery of an AAV vector encoding the *Rs1h* cDNA restores Rs1h protein production in all layers of the retina [242], restores ERG b-wave with a normal electropositive waveform to near-normal levels up to five months post-injection [243]. Thus gene replacement therapy directed towards photoreceptors and inner retinal cells can mediate long-term therapeutic benefit, as demonstrated by AAV-mediated gene delivery in the *RPGRIP*$^{-/-}$ mouse and the *Rs1h*-

deficient mouse. However, studies using other models of RP caused by mutations in photoreceptor-specific genes have shown that gene replacement alone is not always sufficient to mediate long-term functional rescue.

Gene replacement has been shown to ameliorate the structure and function of rod photoreceptors in the *Prph2*$^{Rd2/Rd2}$ mouse. The lack of rod outer segments can be restored effectively; long-term persistence of newly-formed outer segments has been shown [244], with some structural rescue detectable 42 weeks post-treatment. This is achieved by rAAV2-mediated delivery of the *Prph2* gene driven by a bovine rhodopsin promoter (AAV.Rho.Prph2), and the functional benefit following treatment with this construct has been demonstrated [245]; b-wave amplitudes from treated eyes reach a maximum of 140mV, and resemble the *pattern* (but not absolute amplitude values, which range from 350- to 450 μV) of traces from a wild-type eye in terms of a reduction in b-wave latency on increased stimulus intensity. However, this restoration of function is seen to be transient when electrophysiological function is assessed at longer-term time points [246]; improvement in function peaks six weeks after treatment, and b-wave amplitudes decrease from this time point onwards, although treated eyes retain statistically significantly improved function in comparison to untreated contralateral control eyes until 14 weeks post-injection. Following this time-point, AAV-Prph2 treated eyes show no significant improvement in their b-wave amplitude over uninjected internal controls [246]. These results show that although retinal function can be improved using AAV.Prph2 therapy, this improvement is transient. This could be attributable to several factors.

A possible cause of the transient nature of functional improvement, despite newly formed outer segments persisting over time, is that the viral titres used to treat the *Prph2*$^{Rd2/Rd2}$ mouse in previous studies were insufficient to transduce a large enough proportion of the retina, or that the induction of outer segment formation following *Prph2* gene replacement was not early enough. Whilst previous studies examined retinal structure and function following the injection of $1x10^{10}$ genome copies of AAV per ml, titres two log-fold higher ($1x10^{12}$) are now achieved routinely. It has also previously been demonstrated that the quality and quantity of outer segments formed following AAV.Rho.Prph2-mediated gene therapy are significantly better when treatment is administered at early time points; although treatment of

Prph2$^{Rd2/Rd2}$ mice up to 40 days old does result in formation of outer segments, sub-retinal injection at post-natal day 10 results in more and better organised OS [244]. It may be, therefore, that using higher titre virus, and by intervening earlier using sub-retinal injection of AAV.Rho.Prph2 at post-natal day seven, better rescue can be achieved (see **Chapter 4**). Another reason for the transient nature of functional recovery in the *Prph2*$^{Rd2/Rd2}$ mouse may be that the onset of transgene expression may not be early enough to rescue large numbers of cells from apoptosis; the AAV serotype used in previous studies, AAV2, is known to take 6 weeks before mediating high levels of transgene expression [214], and apoptosis in the *Prph2*$^{Rd2/Rd2}$ mouse is known to peak at post-natal day 18 [102]. Hence as part of these studies we have evaluated gene replacement using the same expression cassette used in previous studies in the *Prph2*$^{Rd2/Rd2}$ mouse, pseudotyped with an AAV5 capsid (AAV2/5.Rho.Prph2, see **Chapter 4**). It remains likely, however, that correction of the molecular defect in *Prph2*$^{Rd2/Rd2}$ mice does not interfere with the apoptotic cell death mechanism. Thus, delivering functional copies of genes mutated in disease can be of benefit in certain cases. However, gene correction is not always sufficient to mediate long-term therapeutic benefit, particularly in cases where photoreceptor-specific genes are mutated. For this, a strategy that promotes cell survival, independent of the underlying molecular cause of cell death, needs to be employed.

1.12.3 Neuroprotection

As described in **Chapter 1.10**, pharmacological interventions designed to slow and/or prevent photoreceptor loss are inefficient and require multiple administrations of growth factors. Therefore, in order to achieve long-term neuroprotection, sustained neurotrophic factor delivery to the eye has been investigated. PEDF is one growth factor that has been shown to exert a neuroprotective effect in models of retinal degeneration, as evidenced by work using recombinant protein [126]. The transient nature of the rescue in this study was due to the short ocular half-life of the PEDF protein, and viral delivery of the *PEDF* gene could well circumvent this short-lived aspect of the rescue; this will be investigated as

part of the studies presented here. More recent work has shown a neuroprotective effect of PEDF in the RCS rat, with histological analysis showing significant preservation of photoreceptors at four weeks post-injection; in SIV.PEDF-treated eyes a local treatment effect is seen, with twice as many photoreceptors present in protected regions of the retina compared with untreated areas of the same retina [247]. This study also demonstrated a small improvement in electrophysiological function in PEDF-treated eyes, but only at four weeks after injection; average b-wave amplitudes in SIV.PEDF-treated eyes were 47 μV ± 15.3 μV, compared with 5.5 μV ± 3.9 μV in SIV.lacZ-treated control eyes. Thus some neuroprotective nature of PEDF has been demonstrated, but this is yet to be validated in other RP models.

The most extensively studied neurotrophic factor in the context of viral vector-mediated neuroprotection is ciliary neurotrophic factor (CNTF). CNTF is known to play a role in retinal development, where *CNTF* expression causes a repression of rod development from photoreceptor precursor cells [248]. However, the expression of CNTF receptor on photoreceptor precursors is lost as the rod cells mature, such that in the adult retina photoreceptors do not express CNTFRα. Nonetheless, as CNTF has been shown to protect both retinal neurones [124,249,250] and glial cells elsewhere in the CNS [251] from cell death, CNTF has been used in attempts to protect photoreceptors from apoptosis. As an example of this approach, the *rd* mouse has been shown to benefit from adenovirus-mediated expression of the gene encoding CNTF [252]. This study, however, only looked at outer nuclear layer thickness up to 18 days following treatment; at this very short-term time point, prolonged survival of photoreceptors was demonstrated, but neither the long-term morphological effects of this treatment, nor the functional consequences, were studied. Liang *et al.* subsequently used two transgenic rat models that carry mutant versions of the *Rhodopsin* gene (P23H and S334-Ter), as well as the *Prph2*$^{Rd2/Rd2}$ mouse, to test rAAV-mediated CNTF delivery [253]. They showed that intravitreal virus administration does allow preservation of photoreceptor cells (treated eyes retain up to four layers of photoreceptor nuclei in the ONL 6 to 8 months post-treatment, compared with 1-2 layers in untreated eyes), but that it also leads to a deleterious effect on retinal function in both rats and mice; CNTF-treated animals showed reductions in ERG b-wave, although the b-waves that were recorded appear too small to be reliable (average amplitudes of below 10 μV are reported, which in our

experience is too low to assess the difference between treated and untreated eyes). Other rodent models of RP have been used to study the efficacy of CNTF-mediated neuroprotection. A study in mice expressing a *Prph2* transgene with a P216L mutation also showed a reduction in retinal function following *CNTF* gene delivery [254]. A dose-dependent component to the retinal toxicity was proposed by the authors; they found that expression of *CNTF* using the stronger chicken β-actin promoter (CA)caused a greater reduction in ERG b-wave than when the weaker CMV promoter was used. The CA-driven CNTF expression caused a 74% reduction in b-wave amplitude, whereas the CMV-driven gene caused a 54% drop in b-wave. This was despite a greater number of cells being preserved in the outer nuclear layer, as seen on histology, following CA-driven expression. The authors also reported a re-modelling of the outer retina, whereby cells in the outer nuclear layer had more uncondensed euchromatin and other alterations to nuclear morphology in cells of the inner nuclear layer that are indicative of a change in gene expression patterns in these cells [254]. These data suggested that expressing *CNTF* at inappropriately high levels may be causing the associated functional detriment. A subsequent study investigating the deleterious effects of AAV-mediated *CNTF* delivery showed that normal retinal function is compromised, as well as demonstrating the deleterious effects of CNTF in $Prph2^{Rd2/Rd2}$ eyes treated with AAV-mediated *Prph2* gene delivery [255]. Following sub-retinal injection of AAV.CMV.CNTF in $Prph2^{Rd2/Rd2}$ mice, the small residual b-wave recorded (normally around 60 μV at six weeks post-injection) is reduced by 50%. Indeed, when AAV.CMV.CNTF was used in combination with delivery of AAV.Rho.Prph2, previously shown to improve b-wave amplitude, no improvement in ERG was seen over internal controls; eyes treated with the combination of viruses and those left untreated both had average b-wave amplitudes of around 60 μV. This suggested that damage induced by *CNTF* expression negates any therapeutic effect of *Prph2* gene delivery. Furthermore, AAV-mediated *CNTF* delivery to wild-type eyes leads to loss of function, indicating an impairment of retinal function induced by overexpression of CNTF [255]; wild-type CBA mice showed up to 70% loss of ERG b-wave in eyes treated with AAV.CMV.CNTF when compared with untreated contralateral control eyes. This loss of function was concurrent with a re-modelling of the inner and outer retina that was similar to that seen previously by Bok *et al* [254], and the damage seen after CNTF treatment may originate in the INL. With the neuroprotective effect of CNTF on photoreceptors known to be exerted via the Müller

ganglion cells and the amacrine cells in the inner nuclear layer [92], and with these cells known to be responsible for the generation of the ERG b-wave [256], the remodelling of the inner retina may be involved in the functional damage reported. It is worth noting that studies using encapsulated cell technology (ECT) devices engineered to secrete CNTF have been shown to cause similar dose-dependent deleterious effects, although as they have been studied in non-degenerating, wild-type retinae it is difficult to predict what effect this approach would have on retinae undergoing active degeneration. The data discussed above concerning virus-mediated *CNTF* delivery, however, suggests that although CNTF exerts a potent effect on the survival of photoreceptors in retinal degeneration , even eight months after treatment according to some histological data [253], there is a harmful effect exerted by this neurotrophic molecule that reduces overall retinal function. Further work investigating these deleterious effects is presented in **Chapter 5**.

Glial cell-derived neurotrophic factor (GDNF) has also been used as a neuroprotective agent, with AAV-mediated gene delivery used in rats transgenic for the S334 ter-4 mutant version of *Rhodopsin* [257]. The authors showed both histological preservation of the outer nuclear layer and functional improvements in this model; six weeks post-injection, eyes treated with AAV.GDNF had an ONL that was significantly thicker (up to 28.8 μm) when compared with AAV.GFP-treated controls (21.9 μm) and untreated controls (21.4 μm), and significantly increased b-wave amplitudes (up to 360 μV) in comparison to both uninjected (298 μV) and GFP-treated (315 μV) controls. This work demonstrates the potential of GDNF as a neuroprotective agent for retinal gene therapy; to ameliorate or augment function in cells harbouring mutations may allow a transient reversal of phenotype, but to prolong any improvement it is vital to prevent the death of the cells concerned. Using neuroprotection in combination with gene replacement therapy could have a synergistic effect, resulting in more potent treatment strategies for RP in which gene replacement corrects the underlying pathological defect and neuroprotective agents slow or even prevent cell death. Currently, however, further studies using CNTF, GDNF, PEDF and others are needed before neurotrophic factors can be considered for clinical use alongside gene replacement.

1.13 Summary

Thus far, gene replacement therapy in models of retinal degeneration has been shown to mediate effective long-term disease correction in some cases, whilst other models have shown transient structural and functional improvement that needs to be improved upon, and other models still have proven difficult to demonstrate any efficacy of gene therapy at all. Broadly speaking, the more successful gene therapy approaches for retinal degeneration involve correction of molecular defects in the RPE, which allows inherently healthy photoreceptors to function as normal; models of LCA and other forms of retinal dystrophy caused by RPE-specific defects seem to be more amenable to gene replacement therapy, and because lentiviral vectors mediate fast onset of transgene expression in the RPE, there may be greater success in pursuing gene delivery to the RPE to prevent photoreceptor degeneration. There are models of retinal dystrophy caused by mutations in photoreceptor-specific genes that do show effective disease correction following gene replacement therapy (e.g. *RPGRIP*$^{-/-}$ and *Rs1h*$^{-/-}$ mice), and in these models photoreceptors appear to have more subtle defects that are amenable to viral gene therapy. The models that have more severe structural and/or biochemical defects in photoreceptors, such as the *Prph2*$^{Rd2/Rd2}$ mouse, *Rhodopsin*$^{-/-}$ mouse and the *rd1* mouse, seem to be increasingly difficult to treat using gene replacement alone; this may be due to the fast kinetics of photoreceptor loss that only allows for a narrow treatment window using a very efficient viral vector, or it may be that the underlying molecular defects concerned commit cells to apoptotic death involving a mechanism which cannot be interfered with by simply replacing the missing/mutated gene. In such cases, improved viral vectors, along with safer and more potent neurotrophic agents, need to be developed in order to successfully treat photoreceptor-specific disorders resulting in retinal degeneration.

1.14 Aims and Objectives

As discussed above, viral vector-mediated gene replacement therapy has been shown to be partially effective in the *Prph2*$^{Rd2/Rd2}$ mouse, the RCS rat and other

rodent models of retinal degeneration. However, the functional improvements seen tend to be transient. Neuroprotective gene delivery as been shown to prolong photoreceptor survival in certain models of retinal degeneration, but such a factor that is neuroprotective without the serious detrimental effects seen with CNTF remains to be identified. The aims of these studies are to ameliorate the structural and functional outcomes of gene replacement therapy, by developing strategies based on the previous work in animal models of retinal degeneration. Specifically, the following areas have been addressed:

- How can gene transfer to the retina be improved?
    - We have used novel purification methods to produce pseudotyped AAV, which has been evaluated in three different models of retinal degeneration that require fast onset of transgene expression to achieve a therapeutic effect.
    - We have evaluated integrase-deficient lentiviral vectors for their ability to mediate efficient gene transfer to the retina, and investigated whether they can mediate effective gene replacement therapy in comparison to integrase-proficient vectors in two different models of RP.

- Investigating gene replacement in models of RP caused by mutations in photoreceptor-specific genes.
    - Outer segments formed following AAV.Rho.Prph2 delivery do persist over time, but some are malformed and appear dystrophic [244]. It is possible that overexpression of Prph2 is causing this, and so the effects of inappropriate levels of *PERIPHERIN* need to be investigated. For this wild-type mice have been treated with an AAV vector encoding the *Prph2* cDNA, and the long-term impact on retinal function has been assessed.
    - In previous studies, the most reliable improvement in retinal function in the $Prph2^{Rd2/Rd2}$ mouse was achieved following double injections (inferior and superior hemispheres) at a single timepoint (post-natal day 10). As improvements in surgical techniques now allow subretinal

injections in younger pups (as early as post-natal day one), the effect of earlier and repeated injection has been investigated; this will allow us to determine whether transducing a greater proportion of cells in the retina leads to a more substantial functional improvement.

- It is not clear whether individual transduced cells expressing AAV-mediated *Prph2* survive longer than neighbouring cells that do not express viral transgene. We have therefore assessed transduced cells in the outer nuclear layer to determine whether or not they carry a selective advantage over their non-transduced neighbours. This has been achieved by using a bi-cistronic virus that labels *Prph2*-expressing photoreceptors with GFP, allowing us to use this fluorescent marker to ascertain whether transduced cells survive preferentially.
- Thus far we and others have used the AAV2 serotype to deliver therapeutic genes to the eye. With apoptosis peaking in the *Prph2*$^{Rd2/Rd2}$ mouse retina before AAV2-mediated transgene expression reaches its peak, serotypes with different expression profiles may be more suitable for such applications. For this purpose an AAV2/5 pseudotyped viral vector encoding the Rho.Prph2 construct has been produced and tested in the *Prph2*$^{Rd2/Rd2}$ mouse, to assess whether faster onset of gene expression allows an improved level of efficacy of gene replacement.
- AAV2/5-mediated gene replacement therapy in two further models of retinal degeneration has been evaluated, to validate the delivery of therapeutic genes to the retina using pseudotyped AAV.

- Delivery of genes encoding neurotrophic factors to prevent photoreceptor loss has shown that in the case of CNTF, although some protection from cell death is achieved, there are detrimental effects on retinal function. It may be possible to avoid this reduction in function, whilst retaining the neuroprotective effect of CNTF. There may also be alternative neurotrophins that delay photoreceptor apoptosis without detrimental side effects. Specifically, the following areas have been addressed:

- We have investigated whether reducing the dose of CNTF administered reduces the deleterious effects on normal retinal function seen previously, and if so, whether such a dose possesses any neuroprotective properties.
- Other models of RP could be used to thoroughly test the neuroprotective capabilities of CNTF. We have used the *Prph2*$^{Rd2/+}$ heterozygote mouse, which has larger residual electrophysiological function for longer periods compared to the *Prph2*$^{Rd2/Rd2}$ homozygote mouse, and hence is easier to follow up after treatment with neurotrophins. The long-term effects of CNTF expression in this model has been evaluated in terms of structure and function, which will aid the understanding of the detrimental side effects seen in both wild-type and *Prph2*$^{Rd2/Rd2}$ mouse.
- GDNF has been shown to protect the degenerating rat retina in a transgenic model of RP (see chapter **1.12**). We have evaluated this candidate neuroprotective agent by assessing whether it is better tolerated than CNTF in murine retina, and whether *GDNF* gene delivery can prolong and enhance rescue mediated by gene replacement in the *Prph2*$^{Rd2/Rd2}$ mouse and the RCS rat.

2 Materials and Methods

2.1 rAAV plasmid constructs and virus production

2.1.1 Generation of MOPS promoter DNA

Murine genomic DNA isolated from the tail of a wild-type CBA mouse was used to amplify Mouse Opsin (MOPS) promoter DNA, using the polymerase chain reaction (PCR). A fragment of tail tissue was place in Proteinase K Lysis buffer (100 mM Tris @ pH 8.5, 5 mM EDTA, 0.2 % SDS, 200 mM NaCl) with 1 µl Proteinase K enzyme (20 µg/ml, Sigma, Dorset, UK) and incubated at 56 °C for two hours. The lysate was then spun in a 1.5 ml microfuge tube to remove cell debris, and the supernatant was

diluted 1/5 and denatured at 95°C for two minutes before use in the following PCR protocol.

Using nested primers (see Appendix) a 253 bp fragment of genomic DNA from murine chromosome 6 was amplified. 2 µl of the denatured genomic DNA was added to a PCR tube, and the following reagents added: 2µl each forward and reverse primer (10mM, custom-ordered from Sigma-Aldrich, Dorset, UK), 5 µl 10x NH_4 Reaction Buffer (160 mM $(NH_4)2SO_4$, 670 mM Tris-HCI (pH 8.8 at 25°C), 0.1 % Tween-20), 2 µl 50 mM $MgCl_2$,, 1 µl BioTaq polymerase (all Bioline, London, UK) and 1 µl Vent Polymerase (New England Biolabs, Herts, UK) – the final reaction volume was made up to 50 µl using dH_2O. Following an initial denaturation step (5 minutes at 95 °C), 30 cycles of PCR were carried using the following conditions:

- Denaturation – 30 seconds at 95 °C
- Annealing – 10 seconds at 51.5 °C
- Extension – 30 seconds at 72 °C
- Final extension – 10 minutes at 72 °C

The PCR product was then electrophoresed and isolated as described below.

2.1.2 Electrophoresis, gel extraction and ligation of DNA

DNA products were separated on agarose gels with 1 % (w/v) ethidium bromide using a 1 x TBE buffer (diluted 1/10 from 10 X stock solution, 108 g Tris base, 55 g Boric acid, 9.3 g Na_4EDTA). For resolution of fragments lower than 500 bp in size, 2 % (w/v) agarose (VWR International, Leicestershire, UK) was added to the 1 x TBE, for resolution of larger fragments 1 % was added. The agarose was then heated in a microwave oven until the agarose was completely dissolved, and the gel was allowed to set in a plastic mould. A 1 kb DNA ladder (Promega, Southampton, UK) was run to provide size markers, and both the ladder and all samples were loaded using a 6 x Blue/Orange Loading Dye (0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10mM Tris-HCl (pH 7.5) and 50mM EDTA (pH 8.0 Promega, Southampton, UK). Gels were run at 200 mV for 45

minutes, or until the required bands could be resolved. Gels were photographed on an ultraviolet (UV) transilluminator.

Bands required for ligation were excised using a sterile scalpel blade and placed in a 1.5 ml microfuge tube. 450 µl QX1 buffer was added (Qiagen, W. Sussex, UK) to the gel fragment and the tube was incubated at 55 °C until the gel fragment dissolved. 150 µl isopropanol was added, the tube was inverted twice to mix, and the sample was loaded onto a GenElute HP miniprep column (Sigma-Aldrich, Dorset, UK). The column was centrifuged at 12,000 *g* for 30 seconds, the flow-through discarded, and 600 µl Wash Buffer was added to the column. The column was centrifuged (12,000 *g*, 30 s) and the flow-through discarded, following which the centrifugation was repeated to spin out any residual ethanol in the Wash Buffer. DNA was eluted by adding 50 µl distilled H_2O and centrifuging for 30 s at 12,000 *g*. purified DNA was quantified using a Nanodrop spectrophotometer (NanoDrop Technologies, Delaware, USA) recording UV absorbance at 260 nm.

Fragments purified in this manner were ligated by adding insert DNA to vector DNA in a 3:1 molecular ratio. 1.5 µl DNA ligase buffer (50 mM Tris-HCl, 10 mM MgCl2, 1 mM ATP, 10 mM Dithiothreitol, 5 µg/ml BSA, pH 7.5) and 1 µl T4 DNA ligase (both New England Biolabs, Herts, UK) were added, and the ligation reaction allowed to take place for 30 minutes at room temperature.

2.1.3 Amplification of plasmid DNA in bacteria

Transformation of competent cells

LB-agar plates were made by adding the following to 1 litre distilled water: 10 g NaCl, 5 g Yeast extract B, 10 g Tryptone, 14g agar, (all VWR International, Leicestershire, UK). This medium was autoclaved at 121 °C for 15 minutes, allowed to cool to around 50 °C, and then 50 µg/ml ampicillin (Sigma-Aldrich, Dorset, UK) was added. The LB-agar was set in 9 cm diameter plates and stored at 4 °C. For transformation, DH5α™ competent cells (Invitrogen Ltd., Paisley, UK), were thawed

on ice. The cells were incubated with 500 ng plasmid DNA (30 minutes on ice) before heat shock at 42 °C in a pre-warmed water bath for 45 seconds. The transformation mix was spread onto an LB-agar plate. In cases where a blue-white colony selection was required (i.e. when using pGEM®-T Easy plasmids) 50 µl of X-gal solution (20 mg/ml) was spread over the plate before addition of the transformation mix. Plates were incubated overnight at 37 °C to allow the growth of ampicillin resistant colonies.

Amplification and recovery of recombinant plasmid DNA

Bacterial colonies from agar plates were inoculated into 5 ml of LB medium (as above for agar plates, without agar) containing 50 µg/ml ampicillin and incubated at 37 °C with agitation for 12-16 hours. For small-scale preparations, nucleic acid was prepared using a QIAprep® Spin Miniprep Kit (QIAGEN Ltd., W. Sussex, UK). For bulk cultures, 200 µl of the 5 ml culture was used to inoculate a further 1 l of LB, which was incubated as above. Plasmid DNA was recovered with a QIAGEN® Plasmid Mega Kit (QIAGEN Ltd.).

2.1.4 rAAV production

Recombinant adeno-associated virus particles were synthesised, using the constructs described above, based on methods previously described [258] .The replicating Herpes Simplex Virus (HSV) amplicon pHAV7.3 was used to provide *rep* and *cap* genes required for virion production in conjunction with disabled infectious single cycle HSV (DISC-HSV) as a helper virus. The pD10-based plasmid constructs described above were transfected in a 1:1 weight ratio with pHAV7.3 amplicon plasmid into Baby Hamster Kidney (BHK) cells. BHK cells were cultured to a density of around 60 % confluence (~10^7 cells per 150mm plate) and were incubated with a mixture of Peptide 6 [(K16)GACRRETAWACG], 30 µg construct DNA and 30 µg pHAV7.3 DNA, and 45 µl Lipofectin reagent (Invitrogen, Paisley, UK) per 150 mm plate. A total of twenty plates was used routinely to produce high-titre rAAV. After 4 h incubation with the transfection mix (made in Optimem medium, Invitrogen,

Paisley, UK), DISC-HSV was added to the cells at 10 infectious units (IU) per cell, and allowed to drive rAAV production over 36 hours. Cells were then harvested by scraping, centrifuged, and three freeze-thaw cycles were carried out with vigorous vortexing in between to ensure sufficient lysis of the cells. After treating the lysate with Benzonase (Sigma, Dorset, UK) to remove DNA persisting in the lysate, and 0.5 % deoxycholic acid (DCA, Sigma-Aldrich, Dorset, UK) to liberate virus particles from cellular membranes, the lysate was filtered through 5 μm and 0.8 μm syringe filters (Millipore, Watford, UK). The rAAV particles were purified using a previously described heparin column protocol [209] by applying the filtrate to a heparin-agarose column that had been pre-washed with 10 ml PBS-MK (phosphate-buffered saline (8g NaCl, 0.2g KCl, 1.44g of Na2HPO4, 0.24g, KH2PO4 in 1 litre distilled water) + 1 mM $MgCl_2$ and 2.5 mM KCl). After washing the column with 10ml PBS-MK + 0.1 M NaCl solution, the virus was eluted in 6 ml PBS-MK + 0.4 M NaCl (discarding the first two ml). The eluate was concentrated into 100 μl using Centricon 10 columns (Millipore, Watford, UK), by applying the eluted virus to the column, centrifuging at 5,000x *g* for 75 mins, washing with 2 ml PBS-MK, and spinning to collect the virus concentrate. Resulting rAAV preparations were stored at -80 °C, and were quantified in terms of genomic titre and, where possible, infectious titre.

2.1.5 Recombinant lentivirus production

The protocol used to produce lentiviral vectors was based on a previously published method [259]. Briefly, 293T cells were seeded onto 175 cm^2 plates at around 60 % confluence. The following day these cells were transfected, using the following protocol. Per plate, the following amounts of DNA were added to 5 ml Optimem (Invitrogen Ltd., Paisley, UK) and filtered through a 0.2 μm filter (Millipore, Watford, UK):

Vector construct	- 50 μg
pMDG	- 17.5 μg
p8.91	- 32.5 μg

1 μl of a 10 mM stock of polyethylenimine (PEI, Sigma 40, 872-7) was added to 5 ml of Optimen per plate and filtered through a 0.2 μm filter. DNA and PEI solutions were mixed 1:1 and left at room temperature for 20 minutes. The cells were washed in

Optimem and then 10 ml of the PEI and DNA were added per plate. The cells were incubated at 37 °C, 5% CO_2 for 4 hours. The media was then replaced with D10 medium (. 24 hours after the transfection the medium was removed and replaced with 15 ml complete DMEM per plate. The virus was harvested the following day, as follows. The medium from the cells was centrifuged at 4000 rpm for 10 minutes and then filtered through a 0.22 μm filter. The filtered medium was transferred to polyallomer ultra-centrifuge tubes (11.5 ml/tube TH641 rotor or 33 ml/tube Surespin rotor). The tubes were placed in the rotor buckets and balanced (including the bucket lids). The medium was ultra-centrifuged at 4 °C, for 2-3 hours at 50,000x *g* (23,000 rpm TH641rotor; 22,000 rpm Surespin rotor). The supernatant was carefully decanted off and 50 μl (TH641 rotor) or 150 μl (Surespin rotor) of serum-free medium or medium used for transduction was added per tube and pipetted three times. Parafilm was placed over the top and the tubes were left on ice for 1 hour. The medium was then pipetted 10 times to resuspend the virus and transferred to a 1.5 ml microfuge tube. The virus was then centrifuged for 10 minutes at 4000 rpm to remove cell debris and aliquoted in 2 ml cryovials. The virus was stored at –70 °C. The DNA present in the virus medium was removed to allow transduction efficiency to be measured by DNA analysis. For this, before aliquoting the virus was adjusted to 10 mM $MgCl_2$ with 1 M $MgCl_2$, and DNAse I (Promega M6101) was added (final concentration 5 u/ml). The virus was incubated at 37 C for 30 min, aliquoted and frozen.

2.1.6 Genomic titre of rAAV

Each batch of recombinant AAV produced was quantified by dot-blot assay to determine the number of genome copies per ml of virus. 1 μl and 5 μl of viral sample were digested with 1 μl Proteinase K enzyme (Sigma-Aldrich, Dorset, UK) in 200 μl Prot K buffer (10 mM Tris pH 8, 10 mM EDTA pH8, 0.5 % SDS) for 30 min at 56 °C. After adding 20 μg glycogen, 21 μl 3M sodium acetate ($NaCH_3CO_2$) and 260 μl 100 % ethanol, the digested virus was incubated at -20 °C for 30 min and centrifuged at 14000 rpm for 10 min. The precipitated viral DNA was then washed in 70 % ethanol and centrifuged again at 14000 rpm for 5 min and resuspended in 200 μl 0.4 M NaOH + 10 mM EDTA. A dilution series of plasmid DNA was prepared, with 10^{12} to 10^5 molecules in 0.4 M NaOH + 10 mM EDTA; these would act as standards on

the blot. Viral samples and the standards were denatured at 95 °C for 2 min, and then placed on ice. A 12 cm x 8 cm piece of nylon transfer membrane (Amersham Biosciences, Bucks, UK) was placed in a Dotblot apparatus (BioRad). Wells that were to be used were filled with 200 µl sterile H_2O and a vacuum applied to the apparatus. Samples and standards were then placed in the wells and applied to the membrane under vacuum. The wells were washed with 200 µl 0.4 M NaOH + 10 mM EDTA; the DNA was cross-linked to the membrane on a sheet of 3 MM Whatman paper (VWR, Leicestershire, UK) by incubating at 37 °C for twenty minutes. A hybridisation bottle was pre-heated in a hybridisation oven at 65 °C, together with 50 ml Church mix (0.24 M SDS, 2 ml 0.5 M EDTA, 0.34 M Na_2HPO_4, 0.16 M Na_2HPO_4). The blot membrane was placed in water and transferred to the pre-heated bottle and pre-hybridised for 30 min. During this incubation the DNA fragment to be used as a probe for the blot was isolated from an agarose gel and DNA isolated in QX1 buffer (Qiagen) using mini-prep columns. The isolated probe DNA was resuspended in 65 µl of water, and 34 µl was placed in a fresh microfuge tube and denatured at 95 °C for two minutes. After incubation on ice for 2 minutes, the tube was centrifuged briefly to collect the evaporated liquid from lid. The following reagents were added from the NEBlot Phototope Kit (New England Biolabs, Herts, UK), in order: 10 µl 5x Labelling mix (contains random octomers), 5 µl dNTP mix (contains biotin-dATP), 1 µl Klenow. The probe was ncubated at 37 °C for between 1 hr and overnight, as convenient. The biotinylation reaction was terminated by adding 5 µl 0.2 M EDTA, pH 8. To precipitate the probe, the following was added: 5 µl 3 M sodium acetate, 150 µl 100 % ethanol; this was incubated at -20 °C for 20 minutes. After centrifugation at 14000 rpm for 10 minutes, at 4 °C, the precipitated probe was washed with 70 % ethanol, centrifuged and resuspended in ~20 µl TE or water. 5 µl was to probe blot, the rest was reserved at -20 °C.

5 µl of purified probe DNA was added to the hybridisation bottle containing the blot, and was allowed to hybridise to the samples and standards overnight at 65 °C. The Church mix containing the probe was discarded and he blot was washed three times with 50 ml 33 mM sodium phosphate buffer at pH 7.2 (a 1 M stock of 0.68 M Na_2HPO_4 and 0.32 M Na_2HPO_4 was diluted 1 in 30) for five minutes per wash. The blot was then processed using the Phototope-Star Detection kit (New England Biolabs, Herts, UK) as follows: the blot was blocked by placing in small plastic tray

and adding Block Solution (5% SDS, 125mM NaCl, 25mM sodium phosphate @ pH 7.2) – 20-30 ml to cover blot. The blot was incubated at room temperature on a shaking platform, and the block solution was drained. The blot was then incubated with Streptavidin, diluted 1:1000 in block solution (15 to 20ml in total). Streptavidin solution was then drained. After washing twice for 5 minutes each with Wash Solution I (which is Block solution diluted 1/10), the blot was incubated with biotin-conjugated alkaline phosphatase. diluted 1:1000 in 20 ml Block Solution. After 5 minutes at room temperature, with shaking, this solution was drained and the blot washed once with Block Solution, then wice with Wash Solution II (10 mM Tris-HCl, 10 mM NaCl, 1 mM $MgCl_2$ pH 9.5) – 20 ml per wash was used, and all washed were 5 minutes at room temperature with shaking. During second and final wash, CDP-Star reagent was made up from the Phototope Detection Kit (New England Biolabs, Herts, UK). The assay diluent is 25x concentrate, so first this was diluted 1/25 dilution (2.5 ml needed per blot). Then CDP-Star reagent was diluted 1:100 in the 1x diluent (i.e. 25 µl in 2.5 ml). The blot was placed face upwards on a sheet of clingfilm, and the diluted CDP-Star reagent pipetted carefully onto the blot, ensuring area with dots is well covered. The blot was wrapped in clingfilm to ensure no leakage of CDP-Star reagent, and an X-ray film was exposed to the blot to determine viral titre.

2.1.7 Infectious titre of rAAV

A cell-based *in vitro* assay was also used for all vectors encoding a GFP gene to determine how many infectious particles of virus were produced. 293T cells were seeded onto 24-well plates at approximately 50 % confluency, and were infected with a serial dilution of AAV ranging from 1µl to 1/1,000,000th µl of virus (infections were carried out in duplicate). Wells were superinfected with wild-type adenovirus to provide helper functions to the infecting AAV particles, and infectious titre was calculated after two days of incubation at 37 °C by multiplying number of GFP-positive cells in the well with least green fluorescence by the relevant dilution factor.

2.2 Subretinal injection of viral vector

Injections were performed in either CBA mice of 6-8 weeks or in ten-day-old *Prph2*$^{Rd2/Rd2}$ mice as described previously [213]. Surgery was performed under direct retinoscopy through an operating microscope. The tip of a 1.5 cm, 34-gauge hypodermic needle (Hamilton) was guided in-between a cover slip and a rubber sleeve to the sclera of the right eye and then inserted tangentially through it, causing a self-sealing wound tunnel. The needle tip was brought into focus between the retina and the RPE and approximately 2µl of virus suspension was injected to produce a bullous retinal detachment in the superior hemisphere. A second injection was performed subsequently to produce a similar detachment in the inferior hemisphere.

2.3 Electrophysiological analysis of treated mice

ERGs from treated rats and mice were recorded in a standardised fashion at various time points after injection. All animals were dark-adapted overnight (16 hours) and Ganzfeld ERGs were obtained simultaneously from both eyes of each animal in order to provide an internal control. All procedures for recording were carried out under dim red light. Mice were anaesthetised with a single i.p. injection of 0.15 ml of a mixture of Domitor (1 mg/ml medetomidine hydrochloride, Pfizer Pharmaceuticals, Kent UK), ketamine (100 mg/ml, Fort Dodge Animal Health, Southampton, UK) and water in the ratio 1:0.6:84. Rats were anaesthetised with an i.p. injection of 0.15 ml solution of Domitor and ketamine in water in the ratio 2:3:600. The pupils were dilated using one drop each of Phenylephrine 2.5 % and Tropicamide 1 % to each eye. A single drop of 2 % hydroxy-propyl-methyl-cellulose (HPMC) was placed on each cornea to keep it moisturised. ERGs were recorded using commercially available equipment (Toennies Multiliner Vision, Jaeger/Toennies, Germany) after placing corneal contact lens-electrodes (type Henkes, Jaeger-Toennies, Germany) and midline subdermal reference and ground electrodes. Bandpass filter cut-off frequencies were 1 and 300 Hz for all measurements. Great care was taken to keep electrode impedances symmetrical and low (between 5 and 8 kOhm). Single flash recordings were obtained at light intensities increasing from 0.1 mcds/m^2 to 3000 mcds/m^2. Ten responses per intensity level were averaged with an inter-stimulus interval of 5 s (0.1, 1, 10, 100 mcds/m^2) or 5 responses per intensity

with a 17 sec interval (1000 and 3000 mcds/m^2) to try and minimise the dark adapted conditioning flash effect. All data was analysed using the Toennies Multiliner Vision program.

2.3.1 Statistical analysis

For each animal the b-wave amplitude at 100 mcds/m^2 was used for statistical analysis. The b-wave values (a-wave through to b-wave peak) of the treated (right) eye were paired with the untreated contralateral (left) eye to provide an internal control. A paired Student's t-test was used to evaluate significance ($p< 0.05$). This method controls for inter animal variance and test-retest variance present in rodent ERGs.

2.4 *in vivo* evaluation of transgene expression

Eyes injected with virus containing a GFP reporter gene were evaluated *in vivo* for transgene expression using a slit lamp microscope adapted for rodents. Mice were anaesthetised as described above (**2.3**) and the eyes that were to be examined dilated with 1 % Tropicamide; the mouse was then placed on a platform to allow illumination of the eye via the slit lamp. Dilated eyes were kept moist by placing a drop of Viscotears carbomer gel (Novartis, Hants, UK) on the cornea, and a glass cover slip was placed in contact with the gel to aid visualisation of the retina. A light image was first captured using a Leica DC500 digital camera, following which a blocking filter was applied to the light source which allowed visualisation of GFP fluorescence from the retina.

2.5 Histological analysis

2.5.1 Cryosections

At various timepoints mice treated with viral vectors were anaesthetised with a single intra-peritoneal injection of 0.2 ml sodium pentabarbitone (Euthatal). The animal was then perfusion-fixed; the right atrium was opened with a single cut and 10 ml 4 % paraformaldehyde was injected into the left ventricle for pre-fixation of the tissue until most of the blood was exchanged. Eyes were taken and immediately

immersion fixed in 4 % paraformaldehyde for 4 hours. After fixation the eyes were cryoprotected in 10 % sucrose solution for 20 min at room temperature. Eyes were then embedded in O.C.T. (R A Lamb, E. Sussex, UK) and frozen in iso-pentane which had been pre-cooled in liquid nitrogen. Specimens were stored at -80 °C and 12 μm thick sections were cut using a Bright cryostat. Propidium iodide (PI) was added to fluorescent mounting medium (Dako, UK), and this was added to the slides prior to placing a coverslip on top; the PI acts as a red counterstain for nuclear material, allowing identification of cells on the section.

2.5.2 Paraffin sections

Animals were sacrificed according to Schedule 1 of the Animals (Scientific Procedures) Act 1986. After enucleation eyes were fixed in 1 % buffered formalin overnight. Eyes were then placed in 70 % ethanol to dehydrate them, and embedded in paraffin and sectioned at 5 μm.

2.5.3 Immunohistochemistry

Frozen sections were taken as described above, and air-dried for 10 minutes. After re-hydrating the sections in water for five minutes, the sections to be stained were marked with a hydrophobic marker pen and were blocked using 1 % bovine serum albumin (BSA) and 3 % normal goat serum (NGS) in TBS-T for an hour at room temperature. Sections were then incubated with primary antibody diluted in block solution overnight at 4 °C. After washing three times with TBS-T, sections were incubated with secondary antibody diluted in TBS-T for two hours at room temperature. After washing twice with TBS-T sections were counterstained with propidium iodide or DAPI diluted 1/1000 in TBS-T, washed once with TBS-T and mounted in fluorescent mounting medium. For all antibody dilutions, please see Appendix.

3. Improving gene delivery to the retina

3.1 Introduction

Viral gene delivery to the eye was first attempted using a recombinant adenoviral vector encoding a *β-Gal* reporter gene, which resulted in short-term transgene expression in RPE cells and in a few photoreceptors [229,260]. However, this transgene expression is lost over time as the episomal Ad genome is lost, and an immune response to the adenoviral vector eliminates transduced cells [168]. In disease paradigms in which high-level, short-term transgene expression is enough to mediate a therapeutic effect, Ad-based vectors may be of benefit; for the majority of ocular gene therapy applications, however, longer-term, stable transduction of cells is required. As discussed previously, two types of viral vector have shown efficacy in delivering therapeutic nucleic acids to the retina – those based on the adeno-associated virus (AAV) and those based on HIV-1 (lentivirus, see **Chapter 1.11**). Not only is long-term, stable transgene expression achieved using both recombinant AAV [214] and recombinant lentivirus [199], both vectors can restore structure and function to various models of retinitis pigmentosa [204,205,235,238,245,261].

Vectors based on adeno-associated virus are the most widely used viral gene transfer agents in ocular therapy; following extensive work in animal models of retinal disease (reviewed in [215]), an AAV-based vector is currently being used in two clinical gene therapy trial for LCA patients carrying mutations in *RPE65* [262]. As discussed in **Chapter 1.11**, ease of production, a well characterised transduction profile and the highly efficient transduction of both photoreceptors and RPE (following sub-retinal delivery) contribute to AAV2 being the most widely used serotype for retinal gene therapy. However, with the development of pseudotyped vectors, it is possible to transduce more cells, achieve a faster onset and higher maximal levels of transgene expression, and circumvent the possibility of an immune reaction against a serotype that patients may have been previously exposed to [263]. Pseudotyping produces AAV virions consisting of a genome based (generally) on AAV2, encapsidated by

proteins from a different serotype; such hybrid virions can be made because the *cap* gene that encodes viral capsid proteins is provided *in trans* to the transgene cassette during virus production, meaning the *cap* gene from (for instance) AAV4 can be co-transfected with a transgene cassette flanked by AAV2 ITRs, resulting in AAV2/4 being made. Pseudotyped viruses based on AAV2 have different transduction profiles to AAV2 itself following intraocular delivery, as summarised in **Table 3.1**. By exploiting the specific transduction pattern of a given pseudotype, transgene delivery can therefore be targeted to the tissue of interest; for instance, AAV2/4 would be appropriate for RPE-specific expression of a secreted growth factor [264], whereas AAV2/5 mediates transgene expression in both photoreceptors and RPE [265]. Another feature of pseudotyped vectors is the faster onset of transgene expression following sub-retinal delivery when compared with AAV2; previously published data suggests that AAV2-mediated transgene expression takes up to six weeks to reach maximal levels [214], whereas strong, near-maximal levels of reporter gene expression are seen between two and three weeks post-injection following AAV2/5 mediated transgene delivery [265]. Considering that AAV2-mediated gene replacement therapy in the *Prph2*$^{Rd2/Rd2}$ mouse results in transient improvement in retinal function and no slowing of photoreceptor loss, we decided to pseudotype the AAV2.Rho.Prph2 expression cassette [245,246] with an AAV5 capsid, to determine whether faster onset and more efficient transduction kinetics of transgene expression using this pseudotype would allow prolonged improvement in function and a slowing of photoreceptor loss (**Chapter 4**). However, as well as changing the transduction profile of vectors, pseudotyping also alters the biochemical properties of the mature virions produced. Whilst AAV2 can be purified using a heparin-agarose column [209], it has been demonstrated that AAV2/4 and AAV2/5 are both unsuitable for purification using this affinity chromatography strategy [264]. Described in this chapter are two different purification methods used to produce AAV2/5.Rho.Prph2, and *in vivo* evaluation of this pseudotyped virus as a gene replacement vector is described in **Chapter 4**.

Table 3.1: Tropism of pseudotyped AAV vectors following sub-retinal delivery. Taken from Rolling. *et al*, 2004.

	Mouse	*Rat*	*Dog*	*Primate*
rAAV-2/2	RPE+PR	RPE+PR	RPE+PR	RPE+PR
rAAV-2/1	RPE	—	—	—
rAAV-2/4	—	RPE	RPE	RPE
rAAV-2/5	RPE+PR	RPE+PR	RPE+PR	—
rAAV-5/5	RPE+PR	—	—	RPE+PR

RPE, retinal pigmented epithelium. PR, photoreceptors.

3.2 Improved purification of recombinant AAV vectors

Recombinant AAV2 can be purified using a variety of techniques, including caesium chloride density gradients [266], iodixanol gradients [267] and the current method of choice, heparin affinity chromatography [209]. These methods are difficult to scale up for the production of large quantities of virus, and heparin columns are only efficient at purifying AAV2 and not other serotypes. Hence alternative methods for the purification of AAV-based vectors have been developed that are applicable for more than one serotype and are potentially useful for large-scale vector production. A two-column purification protocol that is suitable for AAV2 and AAV2/5 has previously been described, whereby cell lysates from 293T cells transfected with AAV production plasmids (including the transgene cassette flanked by AAV2 ITRs, a packaging plasmid containing the AAV5 *cap* gene, and a plasmid containing HSV helper functions) was firstly bound to a cationic exchange column, followed by further chromatography on an anionic exchange column [268]. To validate this purification system using packaging plasmids, transgene cassettes and tissue culture methods used routinely in our hands, we attempted to optimise the purification of both AAV2 and AAV2/5 using the previously-described methodology.

Two days after transfection of AAV2 production plasmids, cells were harvested by scraping and resuspended in 1 ml per 15 cm^2 plate of 25 mM $NaHPO_4$, pH 7.4/ 20 mM NaCl, 2 mM $MgCl_2$ (Buffer A). The cells were then freeze-thawed three times and prepared for cationic exchange as previously described [268]. We performed the chromatographic steps using an Äkta Prime FPLC system controlled and monitored by PRIMEVIEW software (both Amersham Biosciences, UK), that follows the pH, conductivity (measured in siemens (S) per area, and proportional to salt concentration) and UV absorbance of the flow-through from the columns. We initially made AAV2 using twenty 15 cm plates of 293T cells, and processed the lysates as above. The crude cell lysate was applied to the cationic SP Sepharose column in Buffer A (see above) adjusted to 150 mM NaCl, washed with one column volume (in our case 5ml) of the same buffer, and was eluted from the column using Buffer A containing a linear gradient of NaCl ranging from 150 mM to 500 mM. We followed conductivity and UV_{280} absorbance of the solution that flowed through the column, and were able to obtain traces similar to those published by Brument *et al* [268]. Examples of these traces are shown in **Fig. 3.1A**; note the point at which the elution buffer is added, and the subsequent rise in conductivity and UV absorbance corresponding to eluate that contains AAV2 particles. The eluate was collected in 1 ml fractions automatically, and these fractions were assessed by dot blot to determine at which point in the elution gradient most AAV2 particles were being recovered. Generally we found that AAV2 genome copies were most abundant between fractions 13 and 17 (**Fig. 3.2A**), which correspond to a conductivity reading of between 20 and 26 mS/cm and a concentration of Buffer B of between 55 % and 70 %. However, on occasion we found a peak of UV absorbance immediately following the addition of Buffer B (**Fig. 3.1B**), which corresponded to elution of some virus particles, which was followed by elution of further AAV2 in downstream fractions (**Fig. 3.1B**). This indicated that the purification of AAV particles from the cationic chromatography column was not as reliable as purification using heparin columns, as elution from these columns occurs at a known salt concentration consistently (0.4 M NaCl).

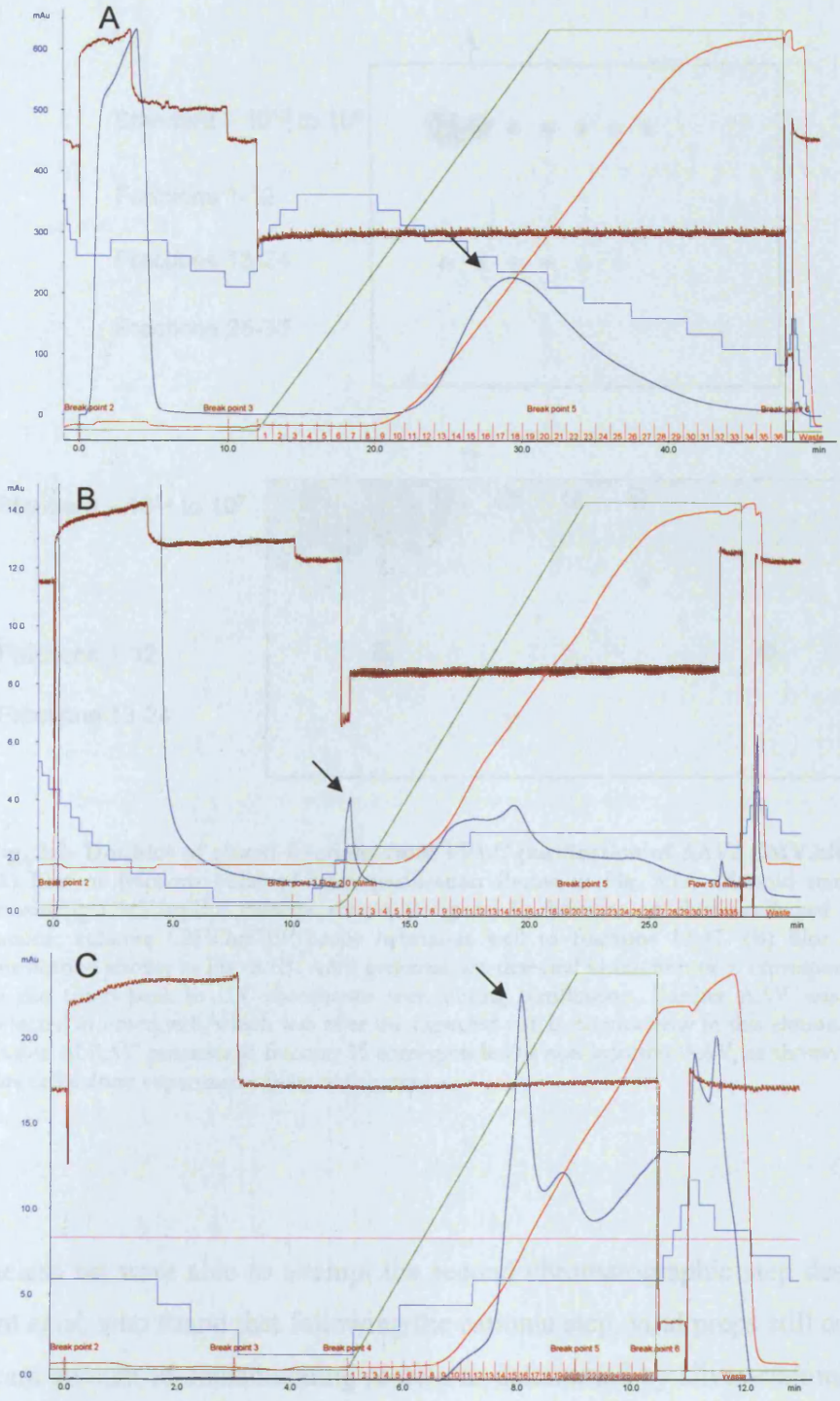


Fig. 3.1: Representative traces from FPLC purification of AAV2.CMV.hfGFP; (A) As the concentration of Buffer B increases, the conductivity (a measure of salt concentration) also increases. A rise in UV absorbance (peak indicated by black arrow) begins shortly after this, corresponding to AAV2 particles being eluted from the column; most AAV2 particles were found in Fractions 13-17 by dot-blot analysis. (B) Plots from a separate purification. A sharp peak in UV absorbance (black arrow) is seen as Buffer B is added, and dot-blot analysis showed that some virus was eluted at this point. AAV2 genomes were also detected in Fraction 8, which was after the expected rise in conductivity in this elution. (C) Plots from second, anionic column; despite a sharp peak in UV absorbance (black arrow), poor recovery of AAV2 was seen on dot-blot analysis, suggesting the UV absorbance was not due to virus being eluted.

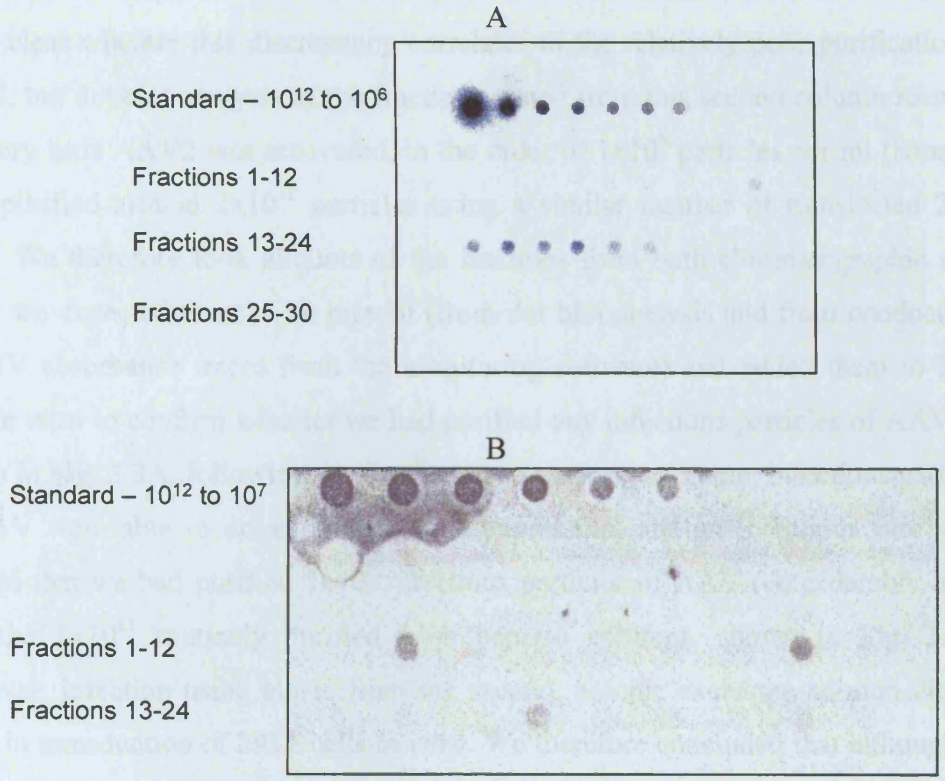


Fig. 3.2: Dot blot of eluted fractions from FPLC purification of AAV2.CMV.hfGFP; (A) Blot of fractions obtained from purification shown in Fig. 3.1A. Plasmid standard containing CMV.hrGFP cassette shown alongside 30 fractions of eluate collected from cationic column; CMV.hrGFP probe hybridises well to fractions 13-17. (B) Blot from purification shown in Fig. 3.1B. AAV genomes are detected in fraction two, corresponding to the sharp peak in UV absorbance seen during purification. Further AAV was also detected in Fraction 8, which was after the expected rise in conductivity in this elution. The elution of AAV genomes in fraction 15 corresponded to non-infective AAV, as shown by *in vitro* cell culture experiments (data not shown).

Nonetheless we were able to attempt the second chromatographic step described by Brument *et al*, who found that following the cationic step, viral preps still contained a significant amount of contaminating protein as determined by silver-staining proteins following SDS-PAGE [268]. We therefore applied the fractions from the cationic exchange column that were identified by dot blot as containing most AAV2 to a Resource-Q anionic exchange column (Amersham Biosciences) in Buffer B (20 mM Tris, pH 8.2, 2 mM $MgCl_2$, 1 mM $CaCl_2$) adjusted to 50 mM NaCl. The bound fraction was eluted using a gradient of Buffer B containing 500 mM NaCl, and we obtained FPLC plots similar in profile to those published previously, but with lower absolute values for UV absorbance (**Fig. 3.1C**); our absorbance peaked at less than 25

mAu, whereas Brument *et al* describe a peak of UV absorbance at around 55 mAu. It is not clear whether this discrepancy correlates to the relatively poor purification of AAV2, but dot blot analysis of the fractions eluted from this second column revealed that very little AAV2 was recovered, in the order of $1x10^6$ particles per ml (Brument *et al* purified around $2x10^{12}$ particles using a similar number of transfected 293T cells). We therefore took aliquots of the fractions from both chromatographic steps where we expected virus to be present (from dot blot analysis and from conductivity and UV absorbance traces from the monitoring software) and added them to 293T cells *in vitro* to confirm whether we had purified any infections particles of AAV. As shown in **Fig. 3.3A**, following the first, cationic exchange column, infectious particles of AAV were able to drive reporter gene expression, and an infectious titre assay showed that we had purified $1x10^7$ infectious particles of AAV (considerably lower than the $1x10^{11}$ routinely purified from heparin columns, shown in **Fig. 3.3B**). However, infection using eluate from the second, anionic exchange column did not result in transduction of 293T cells *in vitro*. We therefore concluded that although we were able to produce infectious AAV2 particles using the first column, attempts to further purify this virus using anionic exchange were unsuccessful. In view of this the AAV2/2 that was used in the experiments described in **Chapter 4** was isolated using a previously described heparin-agarose column chromatography technique that results in reliable and efficient purification.

As this two-step chromatography protocol was designed to purify AAV independent of serotype, purification of AAV2/5 was also attempted using cationic/anionic exchange chromatography. Firstly, we found that co-transfection of our pD10-based AAV2 ITR plasmids with an AAV5 packaging plasmid (kind gift from JA Kleinschmidt, Heidelberg, Germany) did not adversely affect transfection efficiency, and allowed transient expression of reporter gene (hrGFP) *in vitro* as seen during production of AAV2 (**Fig. 3.3C**).

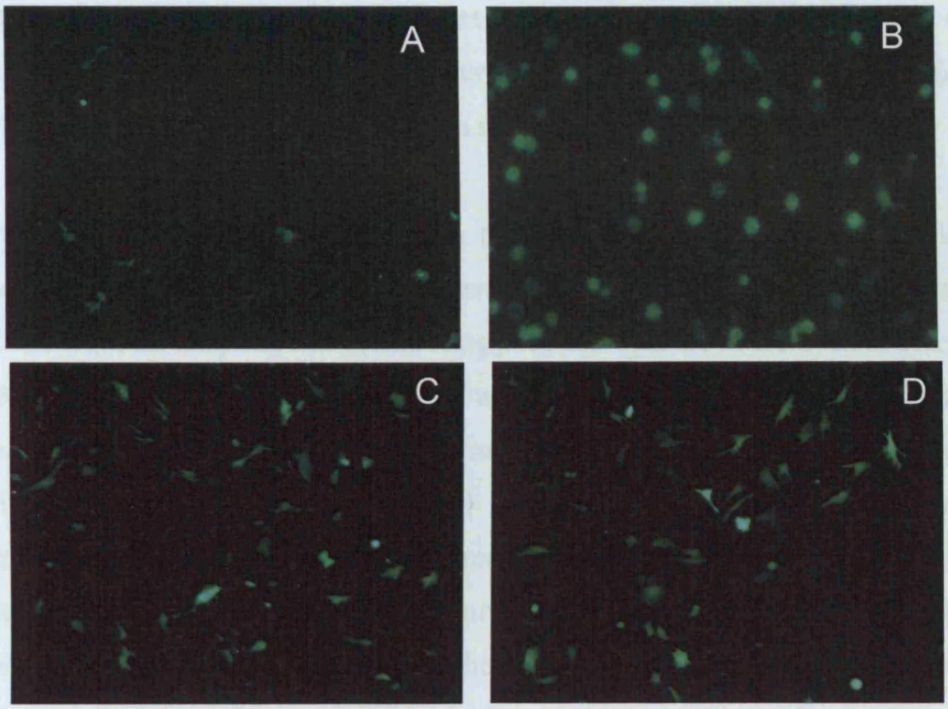


Fig. 3.3: *in vitro* GFP expression using AAV expression cassettes; all images taken using a fluorescent microscope with a 20X objective lens. (A) 293T cells infected with AAV2/2.CMV.hrGFP purified using two-step ion exchange chromatography. Low transfection efficiency is seen. (B) For comparison, 293T cells infected with AAV2/2.CMV.hrGFP purified using heparin column are shown. Cells appear rounded due to superinfection with wild-type adenovirus. (C) Co-transfection of pD10.CMV.hrGFP and AAV5 packaging plasmid does not prevent GFP expression during virus production. (D) 293T cells infected with AAV2/5.CMV.hrGFP purified using sepharose-mucin chromatography shows efficient transduction in the absence of Ad-mediated superinfection.

Lysates from transfected cells were prepared as above, with Buffer A being adjusted to 100 mM NaCl for chromatography of AAV2/5 instead of 150 mM for AAV2 (this is because AAV2/5 has been shown to bind the SP Sepharose column efficiently at this lower salt concentration [268]). Cationic exchange chromatography was again performed using an SP Sepharose column, and the bound fraction was eluted using Buffer A containing a gradient of NaCl up to 100 mM. Fractions were collected and aliquots were analysed by dot blot to assess the efficiency of virus purification. Representative traces from the FPLC system are shown in **Fig. 3.4**; despite a peak in UV absorbance following addition of Buffer B, dot-blot analysis of the collected eluate showed poor purification from the first, cationic column. Nonetheless the fractions that contained AAV genomes were applied to the second, anionic column

and eluted using Buffer B with a gradient of NaCl up to 500 mM. No AAV genomes above the detection limit of the dot blot assay were found. We concluded that using this two-step purification method we were unable to purify sufficiently high-titre AAV2/5, and so an alternative purification method was investigated.

We decided to purify AAV2/5 using a method based on a previously-published protocol that uses mucin coupled to sepharose to purify recombinant virus particles [269]. As we were adapting this methodology for use with the FPLC system described above, the first step was to couple mucin, which is a protein rich in sialic acid, to the sepharose that was to form the matrix of our purification column. This was done by dissolving 240 mg of mucin type 1S in 4 ml ddH_2O and mixing this with 6.0 g of CNBr sepharose that had been washed three times with 50 ml of 1mM HCl. pH was adjusted to 8.0 and the sepharose-mucin mixture left overnight at 4 ^{o}C. To block non reacted groups, the mixture was then washed with 1 M Tris HCl pH 8.0 for 2 hours, and then washed three times with 4 ml 1 M Tris HCl, pH 8 followed by 3 washes with 1 M sodium acetate pH 3-4. The coupled sepharose-mucin was then packed into an XK 16/20 column (Amersham) which was equilibrated with two column-volumes (i.e., 36 ml) Buffer A (20 mM Bis Tris Propane, 20 mM Tris, pH8) containing 45 mM NaCl, using the Äkta prime FPLC system. AAV2/5 was produced using transient transfection in 293T cells as previously described [269], and the lysate applied to the equilibrated column as a 1-in-3 dilution to dilute the salt concentration and allow virus to bind to the column. The column was washed using two further column volumes of Buffer A containing 45 mM NaCl, and virus was eluted using Buffer A containing 450 mM NaCl. Virus produced using this methodology was concentrated using a Centricon-100 column, and by infecting 293T cells *in vitro* (**Fig. 3.3D**) we showed that around $2x10^{11}$ infectious particles of AAV2/5.CMV.hrGFP had been isolated. We therefore used this sepharose-mucin chromatography method to make AAV2/5.Rho.Prph2 and evaluated this as a gene replacement vector as described in **Chapter 4**.

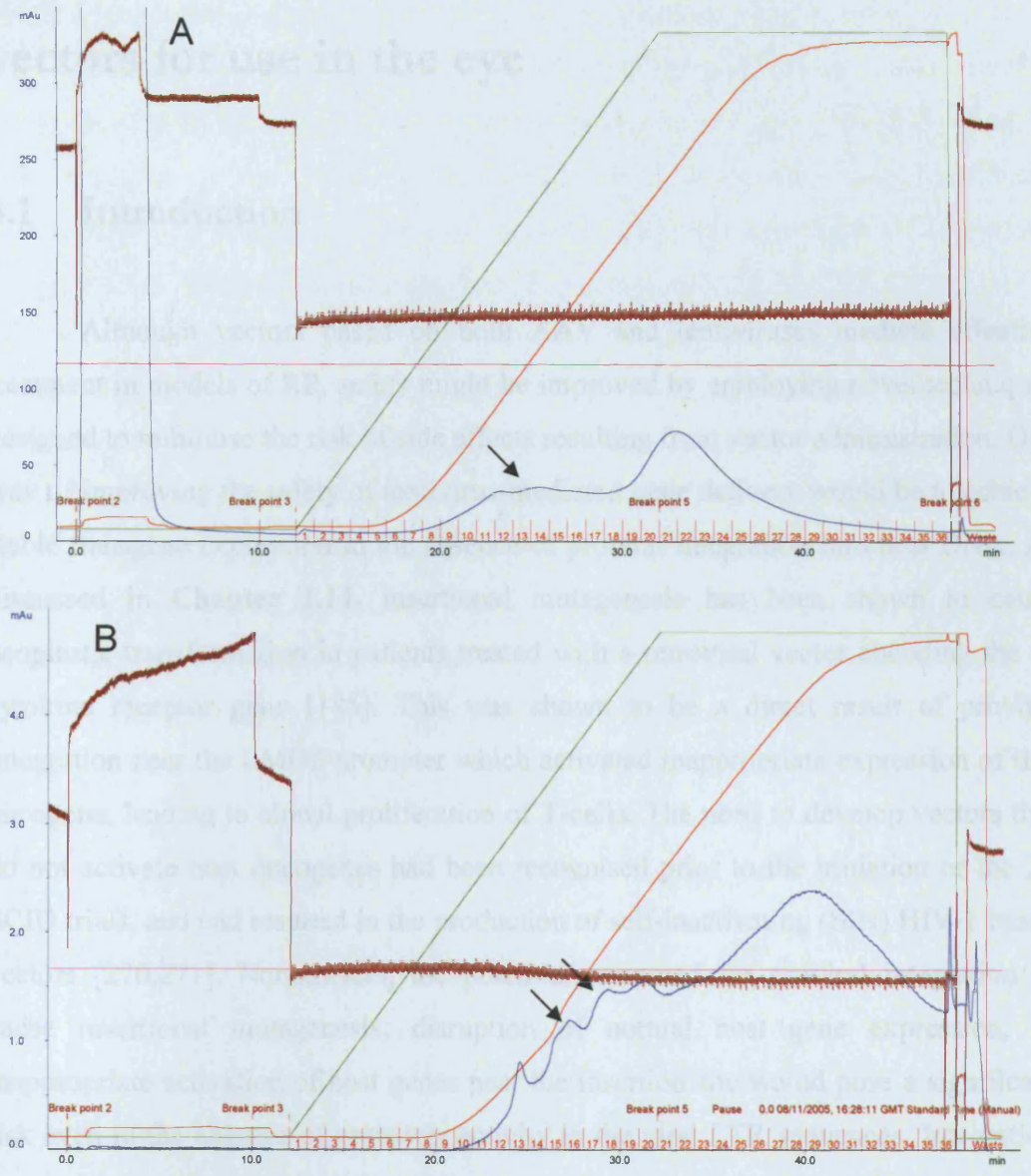


Fig. 3.4: Representative traces from FPLC purification of AAV2/5.CMV.hfGFP; (A) AAV2/5 particles were found by dot blot in fractions 13 and 14, where UV absorbance shows a small increase (small peak indicated by black arrow), and in Fractions 19 and 20. However, the total number of virus particles purified was relatively low compared to purification of AAV2. (B) Plots from the second, anionic exchange column. Despite small, erratic increases in UV absorbance (black arrows, note the Y axis scale in comparison to previous traces), very little AAV2/5 was recovered from the second column.

4 Evaluating integrase-deficient lentiviral vectors for use in the eye

4.1 Introduction

Although vectors based on both AAV and lentiviruses mediate effective treatment in models of RP, safety might be improved by employing novel techniques designed to minimise the risk of side effects resulting from vector administration. One way of improving the safety of lentivirus-mediated gene delivery would be to achieve stable transgene expression in the absence of proviral integration into host DNA. As discussed in **Chapter 1.11,** insertional mutagenesis has been shown to cause neoplastic transformation in patients treated with a retroviral vector encoding the γc cytokine receptor gene [185]. This was shown to be a direct result of proviral integration near the LMO2 promoter which activated inappropriate expression of this oncogene, leading to clonal proliferation of T-cells. The need to develop vectors that do not activate host oncogenes had been recognised prior to the initiation of the X-SCID trials, and had resulted in the production of self-inactivating (SIN) HIV-1 based vectors [270,271]. Nonetheless, the potential remained for proviral integration to cause insertional mutagenesis; disruption of normal host gene expression, or inappropriate activation of host genes near the insertion site would pose a significant risk even in the absence of promoter activity in the viral LTR sequences. Integration of lentiviral provirus DNA is mediated by integrase (IN), encoded by the viral *integrase* gene provided *in trans* during recombinant virus production. This approach allows production of replication-defective lentivirus particles, which contain reverse transcriptase (RT) and IN proteins (to allow efficient proviral synthesis and subsequent integration) without the *rt* and *integrase* genes being incorporated into the vector genome [196]. Mutagenesis experiments were used to identify which protein domains and which amino acid residues within IN are essential for viral integration; these studies identified two classes of IN mutant, referred to as Class I and Class II. Class II mutants have pleiotropic effects on the HIV-1 life cycle, with various processes including Gag/Pol precursor processing, reverse transcription of the viral genome and nuclear entry of the pre-integration complex (PIC) being inhibited; Class

I mutants are specifically blocked at the integration stage, with only the catalysis of proviral integration inhibited whilst all other viral functions remain intact [272]. Thus, the viral genome remains within the host cell as an episome, which is able to direct transgene expression but is lost as a result of successive cell divisions [273]. Despite this, previous studies using a non-human lentivirus vector (based on feline immunodeficiency virus, FIV) showed paucity of transgene expression using a vector carrying a D66V amino acid substitution [202]. Subsequently, an IN-deficient HIV-1 vector that included an origin-of-replication from the SV40 virus was shown to mediate long-term transgene expression *in vitro*. In permissive cell lines that co-expressed the SV40 Large-T antigen, the non-integrated proviral episomes were able to replicate extra-chromosomally and sustain viral transgene expression for up to 60 days [203]. This study demonstrated that episomal proviral DNA can drive efficient transgene expression, and because the majority of cell types that are targeted by ocular gene therapy strategies are non-dividing, we chose to evaluate a Class I IN mutant for its ability to mediate stable transgene expression in the eye.

4.2 Integrase-deficient lentiviral vectors drive efficient reporter gene expression *in vitro* and *in vivo*

We used a mutant *integrase* gene harbouring a D64V amino acid substitution, provided *in trans* to the HIV-1-based expression plasmid, and made recombinant self-inactivating virus using a previously-described method [274]. The integrase-deficient vectors were titre-matched to integrase-proficient vectors, and were evaluated for efficacy of transgene delivery *in vitro* and *in vivo*. The integrase-deficient reporter gene constructs were able to drive stable, long-term transgene expression in murine RPE (**Fig. 4.1**). This was independent of whether they the contained *cis*-acting Woodchuck post-transcriptional regulatory element (WPRE, included in lentiviral vectors to enhance transgene expression), using either the cytomegalovirus (CMV) promoter or the spleen focus-forming virus (SFFV) promoter (both known to drive ubiquitous expression) and with or without the central poly-purine tract (CPPT) from the HIV-1 genome (which aids nuclear transport of the vector genome in host cells) (**Fig. 4.1a**). Reporter gene expression was equivalent in eyes injected with integrase-

deficient vectors as compared with integrase-proficient titre-matched controls; eGFP fluorescence was noted on *in vivo* fundoscopy at seven days post-injection (p.i.) and remained strong until 9 months p.i, the longest time point assessed (**Fig. 4.1a, b**). Six months post-injection, strong eGFP expression was seen in the RPE (**Fig. 4.1c**), consistent with previous studies using integrating lentiviral vectors [199,205]. *In vitro* transduction of human RPE cells was also demonstrated; ARPE-19 cells, an immortalised human RPE-derived cell line [275], showed strong eGFP expression when transduced with integrase-deficient vectors (**Fig. 4.2a, b**), as did cultured primary human RPE taken from an AMD patient following short exposure to vector suspension (**Fig. 4.2c-f**); this demonstrates the potential for *ex vivo* transduction of human RPE, which could be transplanted back into the recipient to mediate a therapeutic effect.

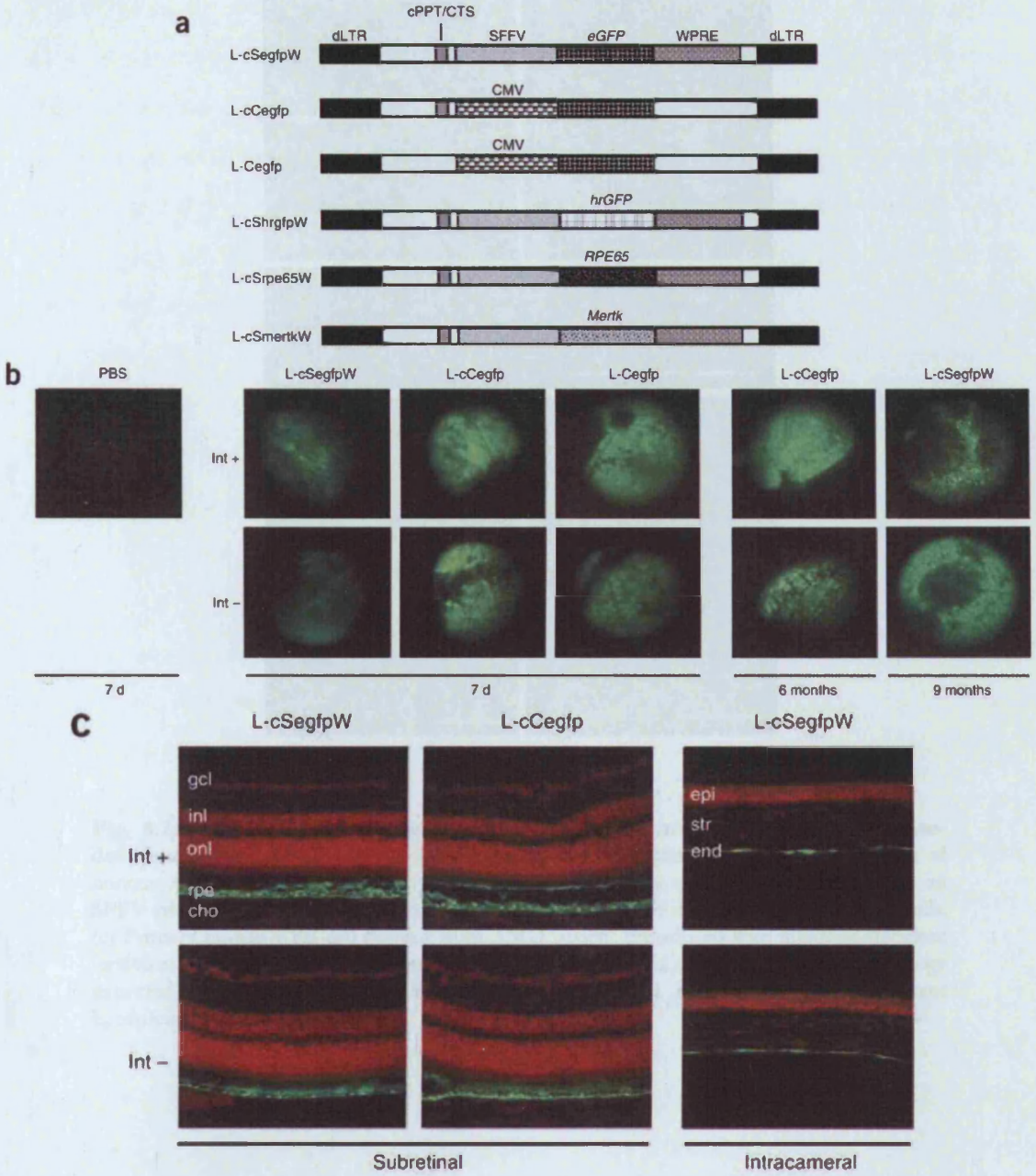


Fig. 4.1: Reporter gene expression in the eye following administration of integrase-deficient lentiviral vectors; independent of vector configuration (a), integrase-deficient vectors were able to mediate strong, long-term GFP expression in the eye as shown by fluorescent fundus photographs taken at various time points (b) and by cryosections counterstained with propidium iodide (red). The route of administration determined viral tropism, with sub-retinal injection leading exclusively to RPE transduction and intracameral injection resulting in corneal endothelial transduction.

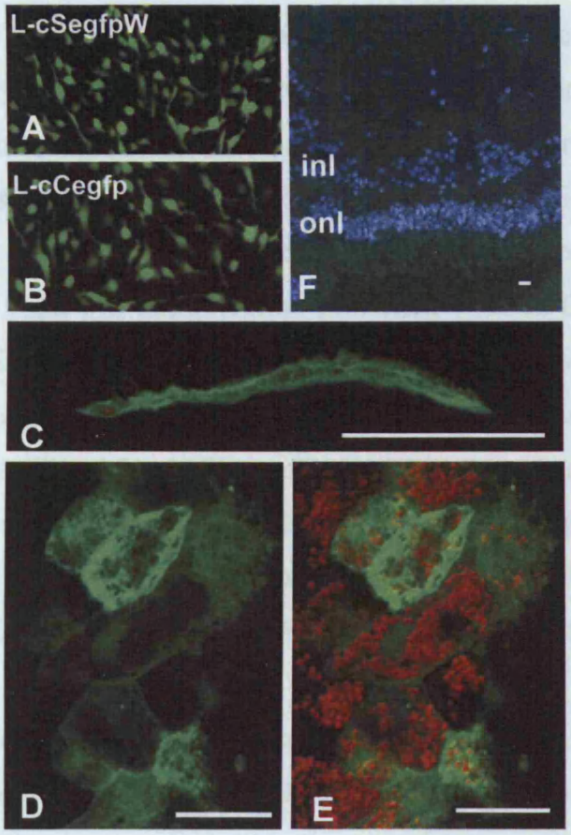


Fig. 4.2: Reporter gene expression *in vitro* following administration of integrase-deficient lentiviral vectors; (a) efficient reporter gene expression following infection of human ARPE-19 cells with an integrase-deficient lentivirus encoding GFP driven by an SFFV promoter. (b) CMV promoter drives similar levels of expression in ARPE-19 cells. (c) Primary human RPE cell derived from AMD patient transduced with integrase-deficient lentivirus encoding GFP. (d) Transduced primary RPE cells show hexagonal morphology expected of macular RPE. (e) Patient-derived RPE cells have many auto-fluorescent lipofuscin granules, shown in red.

4.3 Integrase-deficient lentiviral vectors mediate effective gene therapy in animal models of retinal degeneration

Integrating lentiviruses have been shown to mediate effective gene therapy in rodent models of retinal degeneration [205,276]. We therefore investigated whether our integrase-deficient vectors could improve retinal function in two models of retinal degeneration, the *Rpe65*$^{Rd12/Rd12}$ mouse and the RCS rat, both of which have been previously shown to benefit from viral gene therapy [205,233]. *Rpe65*$^{Rd12/Rd12}$ mice received sub-retinal injection of an integrase-deficient lentivirus encoding the human

RPE65 gene. *Rpe65*$^{Rd12/Rd12}$ eyes treated with the integrase-deficient *hRPE65* vector showed substantially improved ERG waveform, and significantly greater b-wave amplitudes, than untreated contralateral eyes (**Fig. 4.3a, b**). No improvement in ERG was seen following sub-retinal administration of an integrase-deficient virus encoding eGFP (**Fig. 4.3c, d**). Another group of *Rpe65*$^{Rd12/Rd12}$ mice received integrase-deficient RPE65 virus in the right eye and integrase-proficient virus in the left; eyes treated with integrase-deficient virus showed a trend towards higher b-wave amplitudes than eyes treated with integrase-proficient, with improved waveform and more pronounced oscillatory potentials (**Fig. 4.3e, f**). These functional improvements remained until at least two months post-injection, the latest time point studied; at this stage the b-wave amplitudes recorded from *Rpe65*$^{Rd12/Rd12}$ mice treated with integrase-deficient *RPE65* virus were around 50 % of those seen in age-matched wild-type mice.

To validate this novel gene delivery system in another model of retinal degeneration, we used an integrase-deficient virus encoding the *Mertk* gene to rescue the RCS rat; this model of severe early-onset retinitis pigmentosa has previously been shown to benefit from lentiviral gene therapy [205]. Compared with uninjected controls, eyes treated with the integrase-deficient Mertk vector showed improved ERG waveform and amplitude at six weeks post-injection (**Fig. 4.4a, b**); the average b-wave amplitudes recorded from the treated eyes was equivalent to amplitudes seen in the previous study using integrating vectors, being around 100 μV (or around 25 % of wild-type rat amplitudes). One group of RCS rats received sub-retinal injections of integrase-deficient virus in one eye and integrase-proficient virus in the other; both eyes showed a comparable treatment effect, with an average b-wave amplitude of around 120 μV being recorded from both eyes (**Fig. 4.4c, d**). As previously reported, a small, not statistically significant, sham treatment effect was noted using an integrase-deficient virus encoding eGFP (**Fig. 3.8a, b**); this is a result of the injection fluid flushing away the debris layer that accumulates in the sub-retinal space of the RCS rat [205].

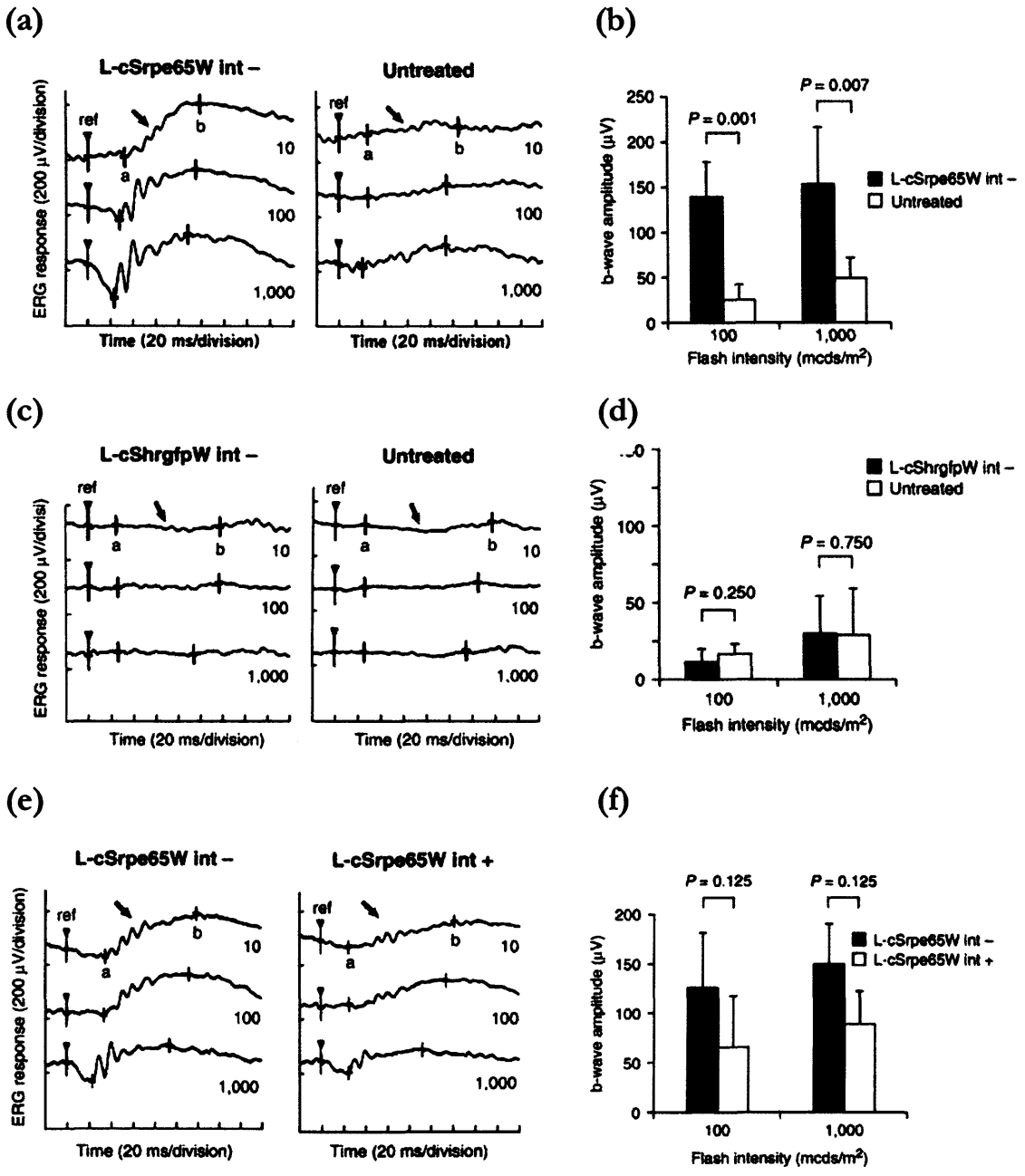


Fig. 4.3: Effective restoration of retinal function following administration of integrase-deficient lentiviral vector encoding human *RPE65*; (a, b) sub-retinal delivery of integrase deficient lentivirus encoding *hRPE65* results in significantly improved ERG waveform and b-wave amplitudes three weeks post-injection compared to untreated contralateral eyes (n = 5). (c, d) no improvement in ERG is seen following administration of an integrase-deficient vector encoding GFP (n = 4). (e) eyes treated with integrase-deficient virus show improved ERG waveform, particularly in more pronounced oscillatory potentials, when compared with eyes treated with integrase-proficient virus; (f) the difference in average b-wave potential was not statistically significant (n = 6).

These data demonstrate that in the absence of proviral integration into host chromosomal DNA, lentiviral vectors are able to drive stable and long-term transgene

expression which can mediate a therapeutic effect in two rodent models of retinal degeneration. This is the first study to demonstrate *in vivo* efficacy of vectors harbouring a mutation in *integrase*, and as such represents an important step in the development of lentiviral gene therapy vectors; in non-dividing cell types the use of episome-derived transgene expression is a promising approach that would allow delivery of therapeutic gene without the risk of insertional mutagenesis seen with integrating viruses.

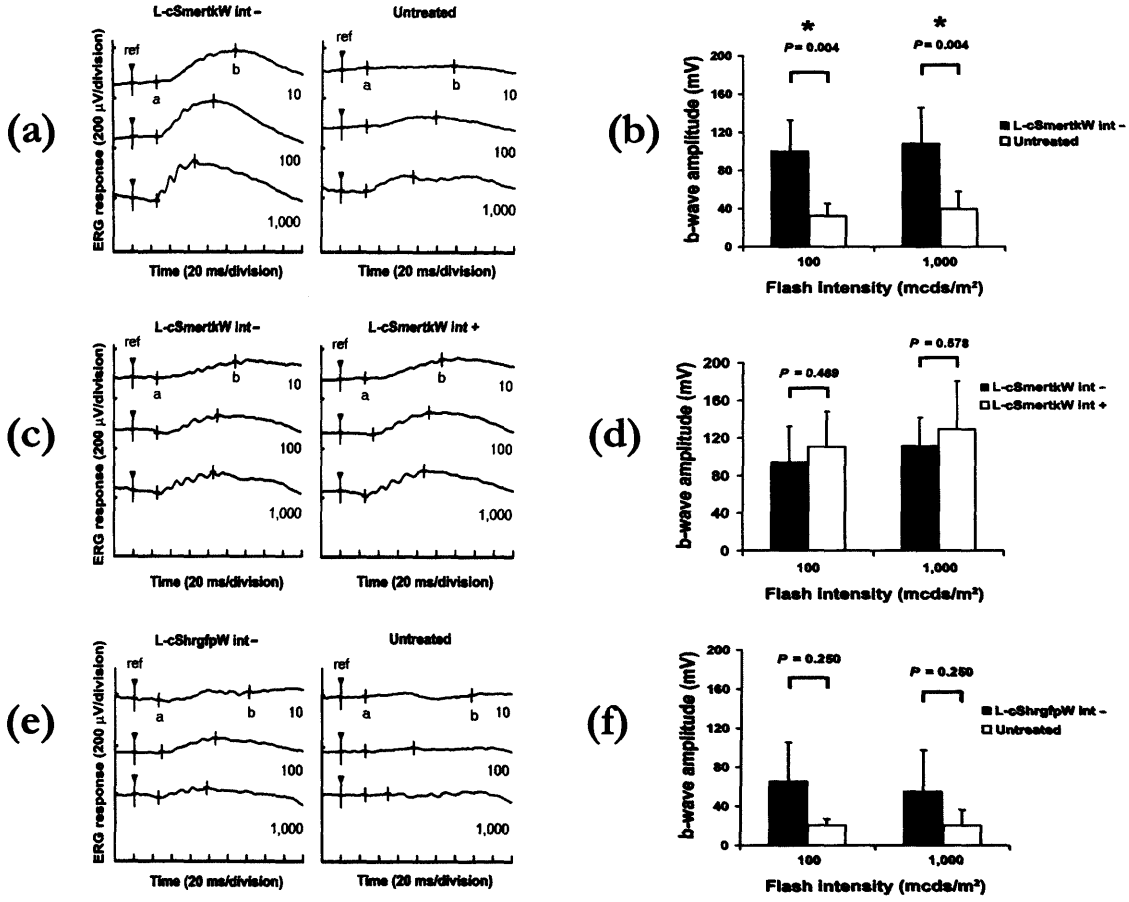


Fig. 4.4: Restoration of function in RCS rats following integrase-deficient lentivirus-mediated gene replacement; Representative ERG traces (a, c, e) and averaged b-wave amplitudes (b, d, f) from RCS rats treated with integrase deficient virus encoding *Mertk*. (a and b) show that following monocular treatment with integrase deficient virus encoding *Mertk* ERG waveform and b-wave amplitude is restored to levels seen in previous studies (n = 6). (c and d) RCS rats injected with integrase-deficient *Mertk* virus in the one eye and integrase-proficient virus in the left eye show that integrase-deficient virus results in equivalent ERG waveform and similar averaged b-wave amplitudes when compared to integrase-proficient virus (n = 6). (e and f) show that there is a small but not statistically significant sham effect on ERG caused by administration of control vector encoding GFP, as described previously (n = 4).

5. Improving gene replacement therapy in rodent models of retinal degeneration

5.1 Introduction

The primary aim of gene replacement therapy is to restore structure and function to cells directly affected by a disease-causing mutation. The earliest studies in gene therapy established proof-of-concept that either lipid-based non-viral vectors [277] or recombinant viruses [278] could be used to deliver genes to host cells. The remaining challenge was to achieve levels of transduction that would produce a therapeutic effect in an *in vivo* model of disease, and ultimately in patients. Whilst this has not been straightforward, much progress has been made in improving the delivery of transgenes to target cells, with improvements made in efficiency of gene transfer, safety of the vectors used and biological efficacy in disease (see **Chapter 1.11, 1.12**).

There have been significant advances in ocular gene therapy since the first studies involving reporter gene transfer to the retina [213,279]. Indeed, many studies have shown physiological disease correction mediated by viral gene delivery in animal models of disease [205,233,235,238,239,245]. Nonetheless, in many cases the improvements in structure and function are transient, largely owing to the failure of gene replacement therapy to delay or prevent photoreceptor apoptosis. Previous studies in rodent models of retinal degeneration have shown that although gene replacement can slow the rate of photoreceptor loss in some cases [205,239], most models fail to show long-term preservation of photoreceptors or amelioration of retinal function following gene replacement therapy [228,246]. As discussed in **Chapter 5**, strategies to enhance photoreceptor survival have focussed on delivery of genes encoding neurotrophic factors to the retina; these have had varying degrees of success, and in the absence of gene replacement therapy often mediate little functional benefit. The aim of the work in this chapter is to investigate whether by improving the efficiency of gene delivery to rod and cone cells, we could increase the duration of improved function in the *Prph2*$^{Rd2/Rd2}$ mouse, the *Rhodopsin* knockout mouse (*Rho*$^{-/-}$)

and the *rd1* mouse by gene replacement therapy. Specifically, we have investigated the long-term effects of AAV-mediated *Prph2* expression in wild-type retinae, and determined whether overexpression of this gene causes any functional deficit. We have also investigated whether using an alternative promoter sequence to drive *Prph2* expression would improve gene replacement therapy and the subsequent functional recovery in *Prph2*$^{Rd2/Rd2}$ mice. Although AAV-mediated gene replacement therapy results in significant functional improvement in the *Prph2*$^{Rd2/Rd2}$ mouse, apoptotic cell death is not slowed as assessed by outer nuclear layer thickness [244]. There are two possible reasons why gene replacement in this model may not lead to a significant reduction in photoreceptor loss. If gene replacement therapy does not confer a selective survival advantage to an individual transduced photoreceptor that expresses *Prph2*, transduced cells would not survive longer than untransduced neighbouring cells. Conversely, if cells expressing *Prph2* do survive longer than their untransduced neighbouring cells, it may be that using pan-retinal ERG or ONL thickness measurements are not sensitive enough to show enhanced survival. In order to investigate the effects of AAV-mediated *Prph2* expression on individual transduced *Prph2*$^{Rd2/Rd2}$ photoreceptors, we needed to mark photoreceptors that express the AAV-mediated *Prph2* cDNA. Hence a construct was developed that included an IRES.GFP sequence downstream of the *Prph2* expression cassette. This enabled *Prph2*-expressing transduced cells to be easily marked with GFP expression; using this construct, the proportion of GFP-positive (and hence Prph2-positive) cells in the outer nuclear layer was quantified, and this analysis was repeated at a later time-point to determine whether transduced cells survive longer than untransduced cells.

We also treated *Prph2*$^{Rd2/Rd2}$ mice with AAV2/5.Rho.Prph2 (described in **Chapter 3.4**) and investigated whether this pseudotyped vector lead to improved ultrastructure, histological preservation and electrophysiological function of photoreceptors when compared to treatment with AAV2/2. In addition we have tested AAV2/5-mediated gene replacement therapy in two other murine models of RP – the *rd1* mouse, which has been difficult to rescue because of the fast kinetics of degeneration, and the *Rho*$^{-/-}$ mouse, which requires high levels of transgene expression in order to achieve functional rescue.

5.2 AAV-mediated overexpression of *Prph2* is well tolerated

Sub-retinal delivery of the AAV.Rho.Prph2 that was being made using the pD10.Rho.Prph2 plasmid available in the laboratory was resulting in poor functional improvement in *Prph2*$^{Rd2/Rd2}$ mice, almost certainly because the 3' ITR in the available pD10.Rho.Prph2 clone had become shortened through recombination as we identified by restriction digestion. Therefore before the following experiments using AAV2/2.Rho.Prph2 could be attempted, the *Prph2* cDNA had to be sub-cloned into pD10-based plasmid in which the ITR sequences were known to be intact. Hence the pD10.Rho.Prph2 plasmid was digested with *BamHI* and *AgeI* to excise the *Prph2* cDNA. Another pD10-based plasmid containing the *rhodopsin* promoter, pD10.Rho.RP2, was digested with *BamHI* and *NotI*, and an oligonucleotide adapter ligated to this to convert the *NotI* overhang to an *AgeI* site. After digesting this plasmid with *AgeI*, the *BamHI/AgeI*-digested *Prph2* cDNA fragment was ligated to give pD10.Rho.Prph2 (**Fig. 5.1**). We verified the new construct by restriction digest analysis to show that the restriction sites missing in the original construct had been restored once the *Prph2* cDNA was sub-cloned into a new pD10 backbone (**Fig. 5.2**). Having shown that this new pD10.Rho. Prph2 clone has the expected restriction sites in the expected orientation, we proceeded to make virus with this plasmid and test it in the experiments described below.

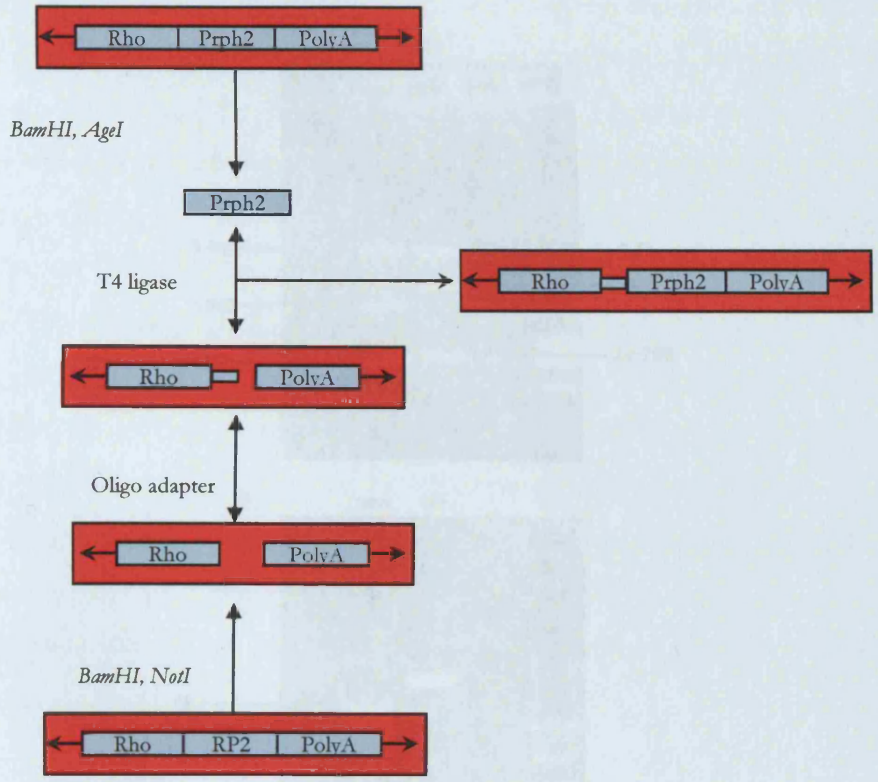


Fig. 5.1: Cloning strategy used to sub-clone *Prph2* cDNA into pD10-based plasmid with intact ITR sequences; the *Prph2* cDNA was excised from the existing pD10.Rho.Prph2 plasmid using *BamHI* and *AgeI* digests. The pD10.Rho.RP2 plasmid was digested with *BamHI* and *NotI*, and an oligonucleotide adapter that changed the *NotI* overhang to an *AgeI* site was ligated. The *Prph2* cDNA was then ligated to this to give a novel pD10.Rho.Prph2 plasmid.

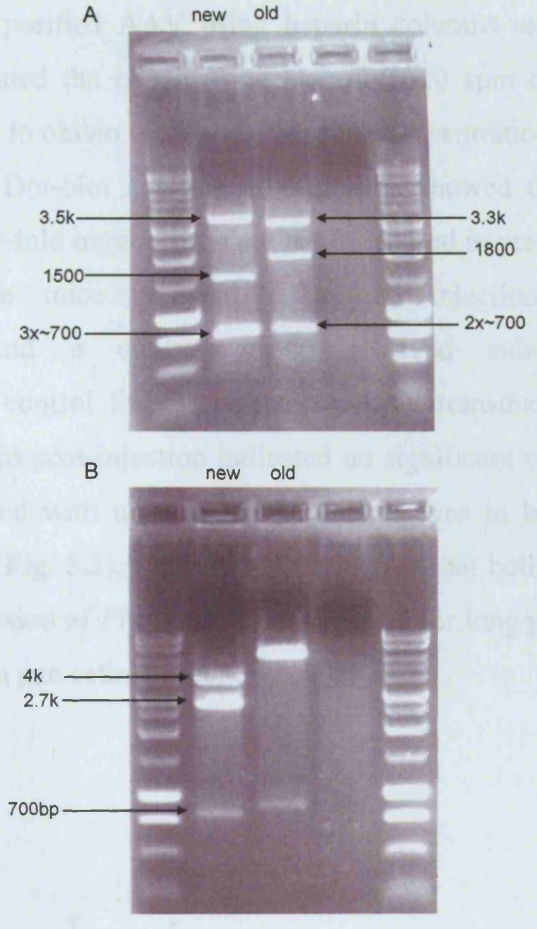


Fig. 5.2: Restriction digest analysis comparing old and new pD10.Rho.Prph2 clones; (A) *SmaI* digests are routinely used by our laboratory to verify ITR sequences; old clone gave fewer bands of (approximately) 700 bp, an unexpected band at 1800 bp, and a smaller band from the vector backbone at 3.3 kbp, whereas the new pD10.Rho.Prph2 clone gave the correct pattern of bands at 3.5 kbp, 1500 bp and three bands at ~700 bp (bands in both lanes at ~400 bp are not visible on this 1 % EtBr agarose gel). (B) A combined digest using *PacI* and *NcoI* enzymes shows that one *PacI* recognition site is missing in the old plasmid, hence there are two bands at ~900 bp and ~6.5 kbp, whereas the new clone gives the expected bands at around 4 kbp, 2.7 kbp and 700 bp.

Previous reports investigating the overexpression of *Prph2* indicated that mice carrying a *Prph2* transgene on a $Prph2^{+/+}$ wild-type background showed no signs of rod or cone dysfunction [280]. However, the suggestion by Sarra *et al* that AAV-mediated *Prph2* overexpression in wild-type mice lead to the loss of photoreceptors was of major concern [244]. In order to investigate the consequences of AAV-mediated *Prph2* overexpression in wild-type retinae, we delivered as high a dose of

AAV.Rho.Prph2 as we could achieve by unilateral sub-retinal injection into wild-type C57Bl/6 mice. We purified AAV using heparin columns as previously described [209], and concentrated the eluate using Centricon-10 spin columns to de-salt the virus suspension and to obtain the highest possible concentration of virus to inject into the wild-type mice. Dot-blot analysis of this virus showed that there were $2x10^{12}$ genomes per ml, 100-fold higher titre than has been used in previous studies [246]. A group of wild-type mice received sub-retinal injections of this high-titre AAV.Rho.Prph2, and a control group received sub-retinal injection of AAV.CMV.GFP to control for the effects of AAV transduction. ERG recordings taken 3- and 8-months post-injection indicated no significant reduction in function in treated eyes compared with untreated contralateral eyes in both *Prph2*-treated and GFP-treated groups (**Fig. 5.3**), a finding that suggests that both injection of high-titre AAV and overexpression of *Prph2* are well-tolerated for long periods of time without detrimental effects on pan-retinal function.

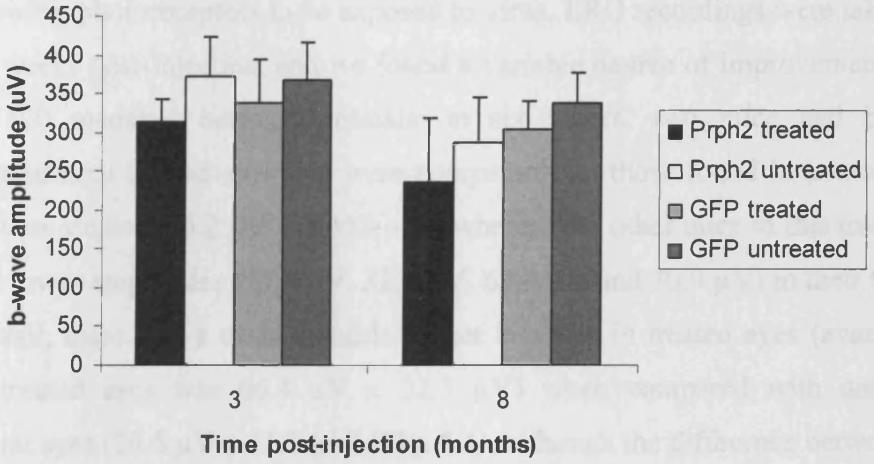


Fig. 5.3: AAV-mediated *Prph2* overexpression is well tolerated: sub-retinal delivery of AAV.Rho.Prph2 to wild-type mice (n=8) does not result in significant reduction in ERG b-wave amplitudes. At three and eight months post-injection, a small reduction in b-wave is seen in eyes injected with AAV.Rho.Prph2 (Prph2 treated) when compared with uninjected contralateral control eyes (Prph2 untreated, p=0.09), which is also seen following sham injection of control vector (GFP treated) compared with uninjected contralateral control (GFP untreated).

5.3 Early and repeated injection of high-titre AAV2.Rho.Prph2 does not enhance gene replacement therapy in *Prph2*$^{Rd2/Rd2}$ mice

Previous studies investigating gene replacement therapy in *Prph2*$^{Rd2/Rd2}$ mice have shown that by treating mice at post-natal day (PND) 10 and again at PND 15, an improvement in retinal function is seen that lasts for over three months post-injection [246]. Having shown that administration of high-titre AAV.Rho.Prph2 ($2x10^{12}$ particles per ml) does not reduce retinal function in wild-type mice (see above), we decided to investigate whether earlier intervention using this virus would result in enhanced functional improvement in *Prph2*$^{Rd2/Rd2}$ mice. Hence double sub-retinal injections of AAV.Rho.Prph2 ($2x10^{12}$ particles per ml) were performed in the dorsal and ventral quadrants at PND 7, earlier than previously attempted in this model and before significant apoptosis is detected in the outer nuclear layer [72]. Five days later sub-retinal injections were performed in previously untreated quadrants of the retina (nasal and temporal) such that approximately 60 % of the retina had a sub-retinal bleb raised allowing photoreceptors to be exposed to virus. ERG recordings were taken six and eight weeks post-injection, and we found a variable degree of improvement in b-wave at 100 mcds/m^2 between animals; at six weeks, two mice had b-wave amplitudes in their treated eyes that were comparable to those found in treated eyes from previous studies (86.2 μV and 113 μV), whereas the other mice in this treatment group had lower amplitudes (27.6 μV, 32.8 μV, 69.0 μV and 70.0 μV) in their treated eyes. Overall, there was a trend towards higher b-waves in treated eyes (average b-wave in treated eyes was 66.4 μV ± 32.3 μV) when compared with untreated contralateral eyes (35.5 μV ± 11.7 μV) (**Fig. 5.4**), although the difference between the treated and untreated eyes was not statistically significant ($p > 0.05$).

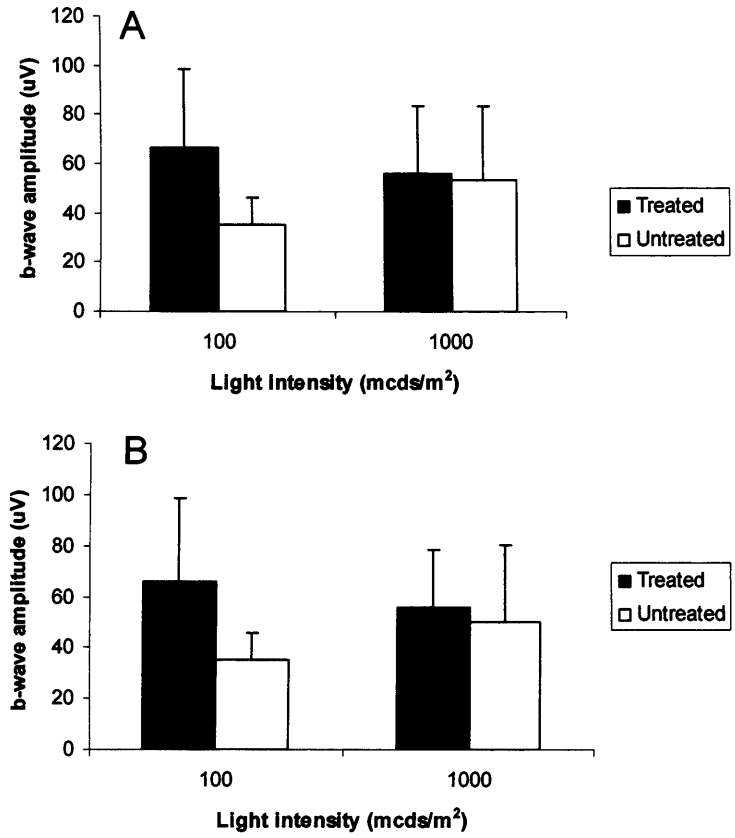


Fig. 5.4: Sub-retinal delivery of AAV.Rho.Prph2 to *Prph2*$^{Rd2/Rd2}$ mice at PND 7 and PND12 does not result in significant improvement in retinal function; (A) Six weeks post-injection, scotopic ERG recordings show that eyes treated with AAV.Rho.Prph2 do not have significantly improved b-wave amplitudes (66.4 μV ± 32.3 μV and 56.35 μV ± 27 μV at 100- and 1000 mcds/m^2 respectively) compared to untreated contralateral eyes (35.5 μV ± 11.7 μV and 53.8 μV ± 31.8 μV) ($p > 0.05$, $n = 7$)). (B) Eight weeks post-injection, there is still no statistically significant rescue of ERG b-wave in treated eyes (65.8 μV ± 33.0 μV at 100 mcd/m2 and 56.2 μV ± 22.6 μV at 1000 mcds/m2) compared to untreated contralateral eyes (35.4 μV ± 10.5 μV at 100 mcd/m2 and 50.5 μV ± 29.8 μV at 1000 mcds/m2, $p > 0.05$).

At 1000 mcds/m^2 we saw virtually no improvement in ERG b-wave across the group; treated eyes had an average b-wave of 56.35 μV ± 27 μV, and untreated eyes had an average of 53.8 μV ± 31.8 μV. At eight weeks post-injection, there was still no statistically significant difference between treated eyes (65.8 μV ± 33.0 μV at 100 mcd/m^2 and 56.2 μV ± 22.6 μV at 1000 mcds/m^2) compared with untreated control eyes (35.4 μV ± 10.5 μV at 100 mcd/m^2 and 50.5 μV ± 29.8 μV at 1000 mcds/m^2, $p > 0.05$). From this we can conclude that the extra trauma caused by two surgical interventions at early time points negates any benefit there may be from having an earlier onset of gene expression; the lack of improvement in b-wave amplitude is less

likely to be caused by the use of higher titre virus, as we have shown that delivery of AAV.Rho.Prph2 at this titre is tolerated in wild-type mice. The fact that some *Prph2*$^{Rd2/Rd2}$ eyes showed an improvement in ERG following early and repeated injection suggests that this treatment can be tolerated in some cases, but that overall the strategy of treating mice at PND 7 and again at PND 12 was liable to causing more damage to the retina and a subsequent absence of the functional improvement seen after surgery at PND 10 and PND 15. In the following experiments involving *Prph2*$^{Rd2/Rd2}$ mice, therefore, we decided to treat *Prph2*$^{Rd2/Rd2}$ mice at PND 10, and to use high-titre AAV to mediate gene therapy.

5.4 *Prph2* expression driven by a novel fragment of the mouse opsin promoter results in efficient transgene expression in rods, but not cones

As discussed previously, it is not clear whether gene replacement therapy in the *Prph2*$^{Rd2/Rd2}$ mouse has any effect on the apoptotic mechanism within individual transduced photoreceptors. To investigate this, a construct with a smaller promoter fragment driving *Prph2* expression was required, in order that a multi-cistronic expression cassette including an IRES.GFP sequence could be accommodated within the 4.8 kb packaging limit of AAV2. As the full-length bovine rhodopsin promoter (referred to as Rho) used in previous studies [244-246] is over 2.2 kb, a novel 235bp fragment of the mouse opsin promoter (referred to here as the MOPS promoter, spanning nucleotides -218 to +17 relative to the start codon of the *opsin* gene) was isolated from mouse genomic DNA (**Fig. 5.5**, see **Appendix** for primer sequences). As this fragment of the MOPS promoter had not previously been validated *in vivo*, this promoter fragment was cloned into an AAV plasmid in place of the CMV promoter to make pD10.MOPS.GFP. An untranslated 1.9 kb fragment of the bacterial *β-Galactosidase* gene was cloned downstream of the SV40 polyadenylation signal, to act as a 'stuffer' sequence; the addition of this sequence meant the final pD10.MOPS.GFP.STUFF plasmid had 3.5 kb of DNA between the AAV2 ITR sequences, which allows the expression cassette to be efficiently packaged into AAV virions.

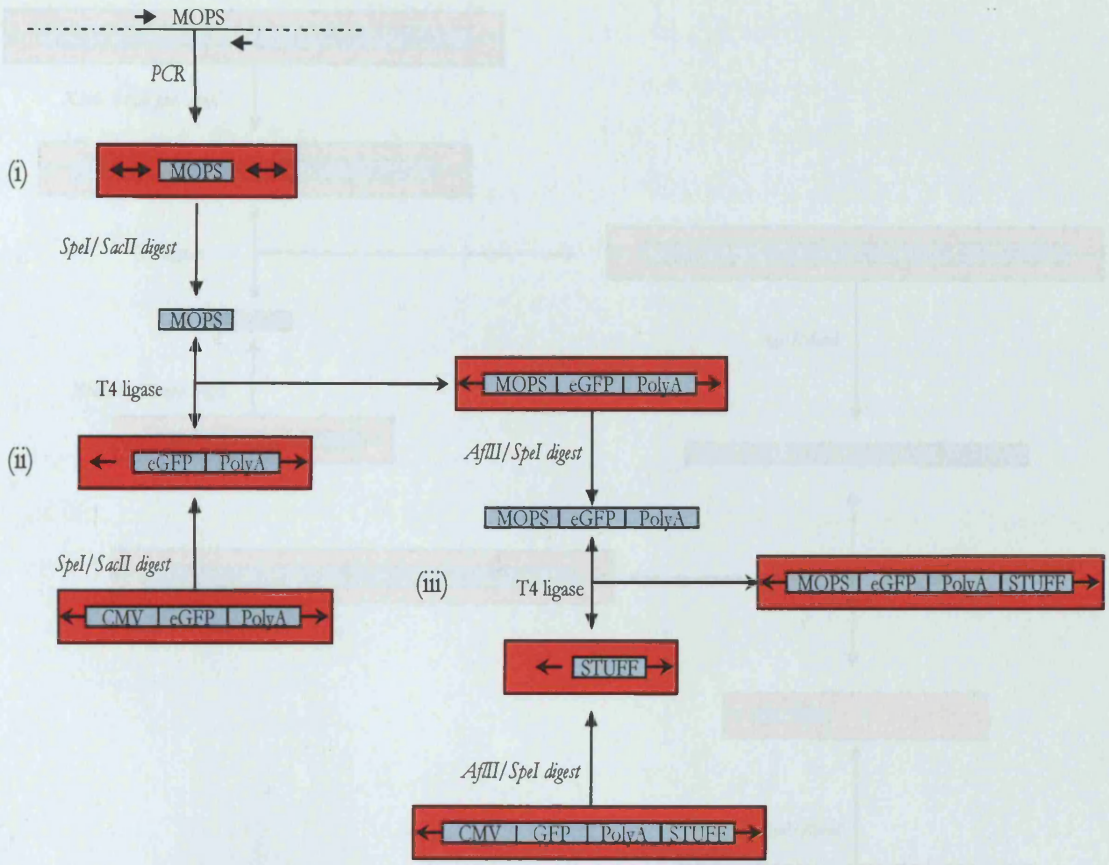
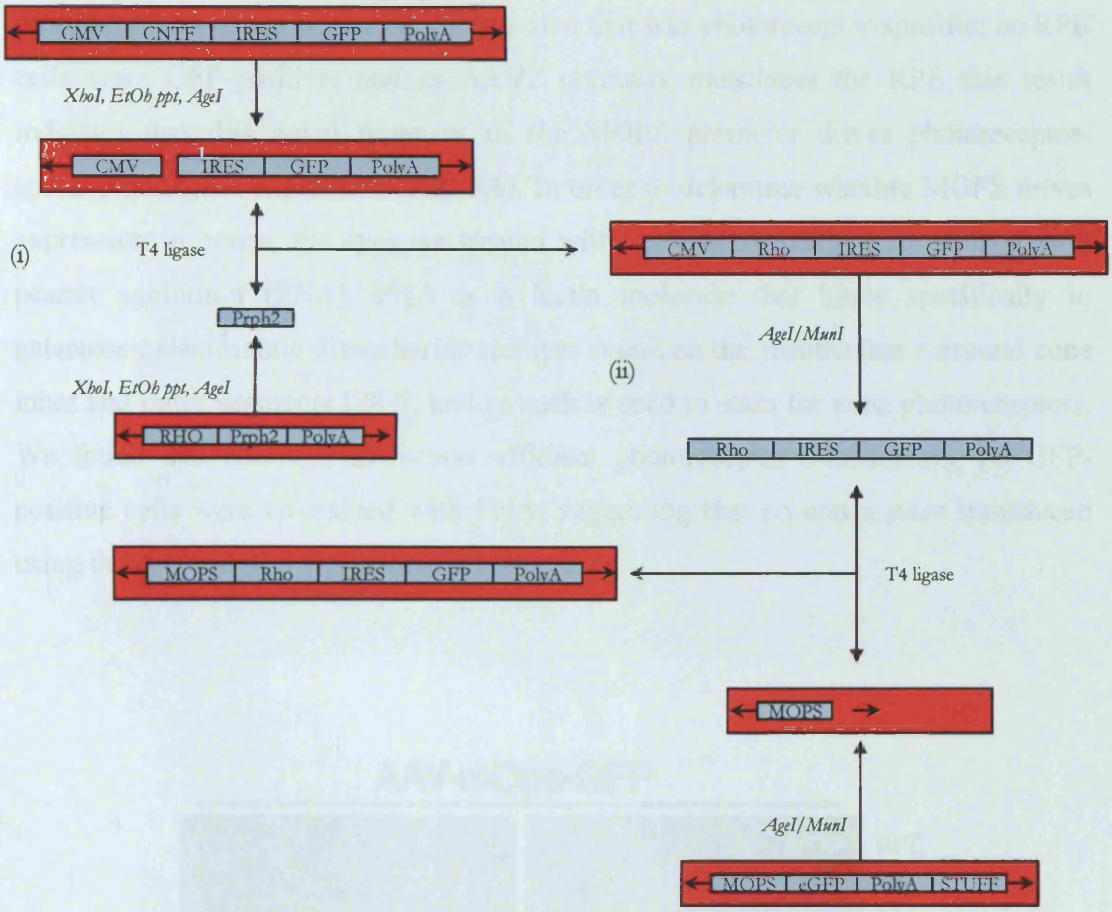


Fig. 5.5: (A) Cloning strategy to used to AAV plasmid containing novel mouse opsin promoter (MOPS) fragment driving GFP; (i) A 234 bp fragment of the mouse opsin promoter was isolated using PCR on murine genomic DNA. This was ligated to the linear pGEM-T easy cloning vector (Promega, UK); *double-headed arrows* indicate multiple cloning sites (MCS). The MOPS promoter was then excised from this vector using *SpeI* and *SacII* restriction enzymes. (ii) The CMV promoter in pD10.CMV.GFP was excised using *SpeI* and *SacII* digests, and the MOPS promoter cloned in its place to generate pD10.MOPS.GFP. (iii) To ensure efficient packaging of this expression cassette into rAAV, pD10.MOPS.GFP was digested with *AflII* and *SpeI* and the expression cassette excised. This was ligated to pD10.CMV.GFP.STUFF that had been digested with *AflII* and *SpeI*, which replaced CMV.GFP.PolyA with MOPS.GFP.PolyA. This resulting plasmid, pD10.MOPS.GFP.PolyA.STUFF, included the non-coding *B-gal* stuffer fragment. *Black arrows* represent AAV2 inverted terminal repeat (ITR) sequences.



(**B**) To use the MOPS promoter to drive *Prph2* and *GFP* expression, (i) The *Prph2* cDNA was excised from pD10.Rho.Prph2 using *XhoI* and *AgeI* restriction enzymes. This fragment was used to replace the *CNTF* cDNA in pD10.CMV.CNTF.IRES by ligating to an *XhoI/AgeI* fragment of this plasmid. (ii) This gave pD10.CMV.Prph2.IRES.GFP.PolyA, from which the Prph2.IRES.GFP sequence was excised using *AgeI/MunI* restriction enzymes; this was used to replace the GFP.PolyA fragment in pD10.MOPS.GFP to give the AAV plasmid pD10.MOPS.Prph2.IRES.GFP.PolyA.

Recombinant AAV2/2 using the pD10.MOPS.GFP.PolyA.STUFF plasmid (AAV.MOPS.GFP) was made as previously described [238], and the titre was determined by dot blot analysis as being $1x10^{12}$ genomes per ml. Wild-type C57Bl/6 mice received sub-retinal injection of 2 µl in one eye as previously described [246], and six weeks post-injection fundus examination revealed strong GFP fluorescence from the retina (**Fig. 5.6**, n = 4). The mice were sacrificed at this time point and assessed histologically to determine whether the MOPS promoter was driving

photoreceptor-specific transgene expression. Fluorescent micrographs of frozen retinal sections reveal strong GFP expression that was photoreceptor-specific; no RPE cells were GFP-positive, and as AAV2 normally transduces the RPE this result indicates that this novel fragment of the MOPS promoter drives photoreceptor-specific transgene expression (**Fig. 5.6**). In order to determine whether MOPS drives expression in cones, the eyes we treated with AAV.MOPS.GFP were stained with peanut agglutinin (PNA). PNA is a lectin molecule that binds specifically to galactose-galactosamie disaccharide residues found on the sheaths that surround cone inner and outer segments [281], and as such is used to stain for cone photoreceptors. We found that although there was efficient photoreceptor transduction, no GFP-positive cells were co-stained with PNA, suggesting that no cones were transduced using the AAV.MOPS.GFP construct (**Fig. 5.7**).

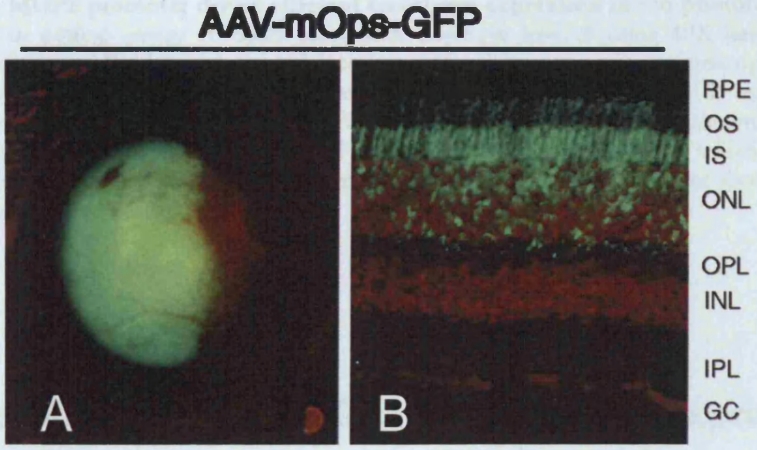


Fig. 5.6: Novel MOPS promoter mediates efficient photoreceptor-specific GFP expression; (A) six weeks after sub-retinal delivery of AAV2.MOPS.GFP to wild-type mice, fundus examination shows transduction of more than 50% of the retina. (B) Representative photomicrograph (20X objective lens) of cryosections from an eye injected with AAV2.MOPS.GFP. Efficient photoreceptor-specific transduction was seen, with absence of GFP expression in the RPE. *Green*, GFP; *red*, propidium iodide nuclear counterstain; RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GC, ganglion cell layer.

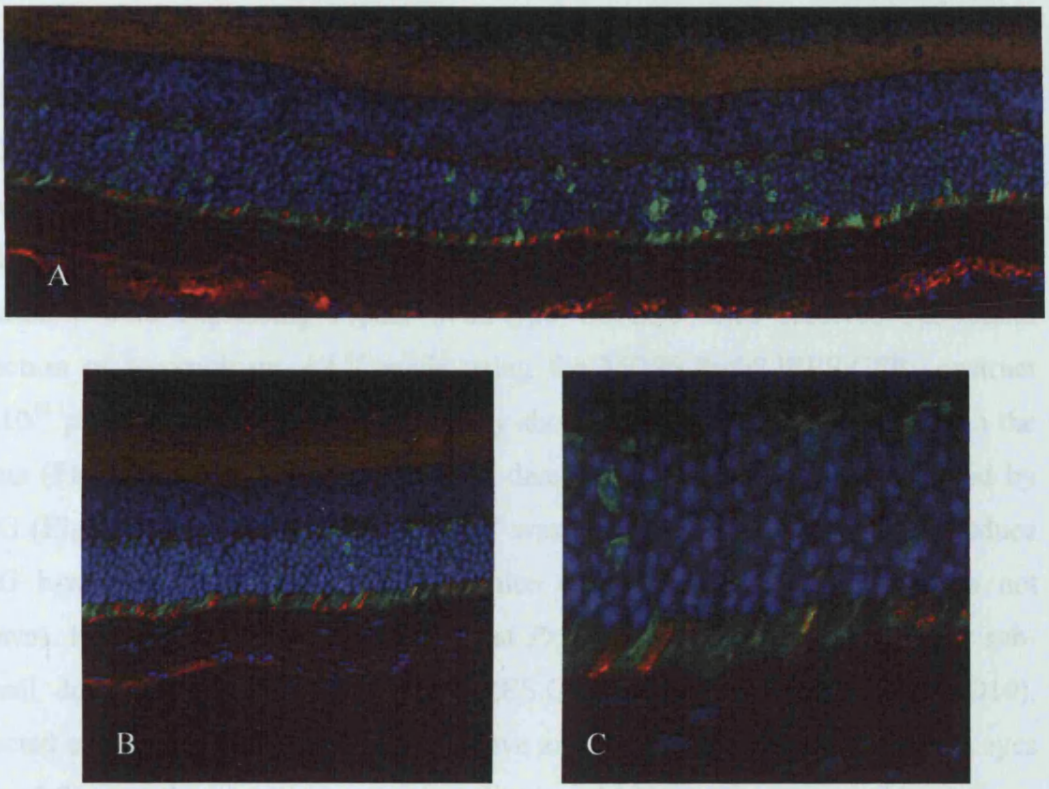


Fig. 5.7: MOPS promoter drives efficient transgene expression in rod photoreceptors but not in cones; (image A taken using 16X objective lens, B using 40X lens, C is a magnified area of B) six weeks post-injection, confocal laser scanning microscopy shows that there is efficient transduction of photoreceptors as evidenced by GFP expression. However, there is no co-localisation of GFP with cone opsin staining (shown in red), indicating that all none of the transduced cells are cones. The only cell in which red and green signals are apparently seen was shown to be two distinct cells in different slices of a Z-stack image.

5.5 Investigating the effect of *Prph2* expression on individual photoreceptors following AAV-mediated gene delivery

In order to follow whether individual photoreceptors survive for longer than untransduced neighbouring cells, we cloned the *Prph2* cDNA downstream of the MOPS promoter described above, and in place of the *β-Gal* stuffer sequence an IRES.GFP sequence was sub-cloned; this gave pD10.MOPS.Prph2.IRES.GFP (**Fig. 5.5B**). The MOPS promoter fragment was chosen as it has been previously validated in an AAV vector in terms of driving effective transgene expression in photoreceptors

[239]. Also, the MOPS promoter fragment, at less than 250 bp, was the only available promoter fragment that was small enough to allow the Prph2.IRES.GFP expression cassette to be packaged into the 4.8 Kbp AAV genome. The IRES was included in order to drive GFP expression and thus mark transduced cells, which by extension were expressing Prph2. Wild-type C57Bl/6 mice received sub-retinal injection of recombinant AAV made using the MOPS.Prph2.IRES.GFP construct ($1x10^{12}$ particles/ml); although fundoscopy showed extensive GFP expression in the retina (**Fig. 5.8 A**), injected eyes showed damage to retinal function as assayed by ERG (**Fig. 5.8 B**). A new batch of rAAV was made, and was shown not to reduce ERG b-wave amplitudes in wild-type mice eight weeks post-injection (data not shown). Hence this virus was used to treat *Prph2*$^{Rd2/Rd2}$ mice. Six weeks after sub-retinal delivery of AAV.MOPS.Prph2.IRES.GFP (surgery performed at PND10), injected eyes had significantly higher b-wave amplitudes than uninjected control eyes (**Fig. 5.9**), and the average b-wave amplitudes obtained were comparable to those seen following treatment with AAV.Rho.Prph2. Four mice were sacrificed and their eyes processed for cryosectioning. In order to determine the proportion of GFP-positive (and hence Prph2-positive) photoreceptors in treated retinae, serial cryosections were taken at 10 μm thickness and at fixed positions in each eye confocal microscopy was used to generate images of equivalent areas in treated sectors of retina (**Fig. 5.10A**). By quantifying the average number of photoreceptors present (propidium iodide counter-stained nuclei, five images per eye), we confirmed that between the two time-points assessed, degeneration of the ONL was taking place; at six weeks post-injection there were 233.7 ± 27.7 photoreceptor cells per field of view, whereas at 12 weeks post-injection there were 174.7 ± 3.7 photoreceptors per field of view (**Fig. 5.10B**, p=0.02). The number of GFP-positive photoreceptor nuclei in the ONL of each image was quantified, and 10 images per eye were analysed in this manner. This gave an average number of GFP-positive nuclei per eye, which was plotted as a percentage of total cells in the same eye (**Fig. 5.10C**). At six weeks post-injection, 48.9 % ± 2.6 % of photoreceptors were GFP-positive (and, by extension, expressing *Prph2*). At 12 weeks there was a small reduction in the percentage of transduced cells, with 41.4 % ± 4 % of photoreceptors being GFP-positive; an unpaired Student's T-test showed that this difference was statistically significant, albeit marginally so (p=0.04). This result indicated that individual photoreceptors that express AAV-mediated *Prph2* do not survive for longer than untransduced

neighbouring cells, and that there may be a small negative effect whereby transduced cells survive less when compared to untransduced cells. Overall this suggests that *Prph2* expression does not confer a selective advantage to photoreceptors in *Prph2*$^{Rd2/Rd2}$ retinae.

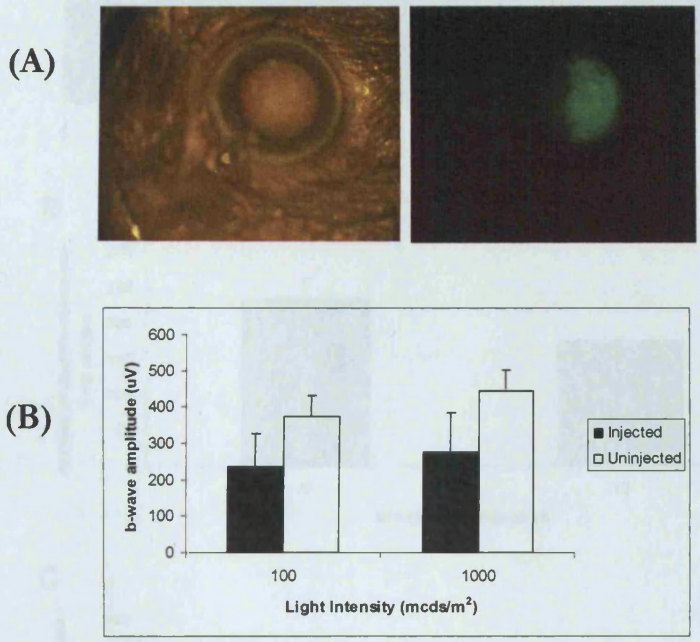


Fig. 5.8: Sub-retinal delivery of AAV.MOPS.Prph2.IRES.GFP to wild-type mice results in strong GFP expression and reduction in function; (A) six weeks post-injection, fundus examination of wild-type mice shows strong transduction of over half the retinal area (left panel, light image; right panel, fluorescent image). (B) Average b-wave amplitudes recorded at 100- and 1000mcds/m2 show significant reduction in retinal function in eyes injected with AAV.MOPS.Prph2.IRES.GFP (235.5 μV ± 92 μV and 276.9 μV ± 90 μV at the respective intensities) compared to untreated contralateral eyes (372.5 μV ± 58.5 μV and 445.3μV ± 108 μV respectively, $n = 6$).

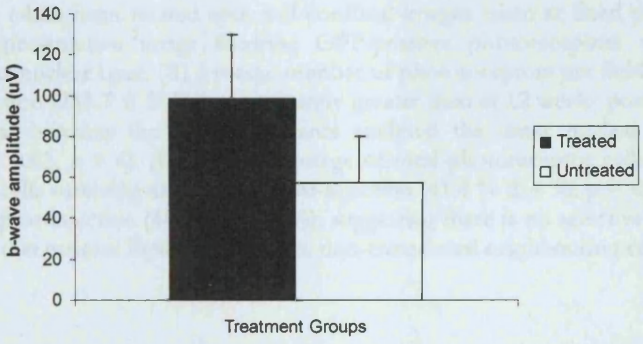


Fig. 5.9: Sub-retinal delivery of AAV.MOPS.Prph2.IRES.GFP to *Prph2*$^{Rd2/Rd2}$ mice results in significant improvement in retinal function; six weeks post-injection, scotopic ERG recordings show that eyes treated with AAV.MOPS.Prph2.IRES.GFP have significantly improved b-wave amplitudes (98 μV ± 31.4 μV) compared to untreated contralateral eyes (57.3 μV ± 22.6 μV) ($p < 0.02$, $n = 8$).

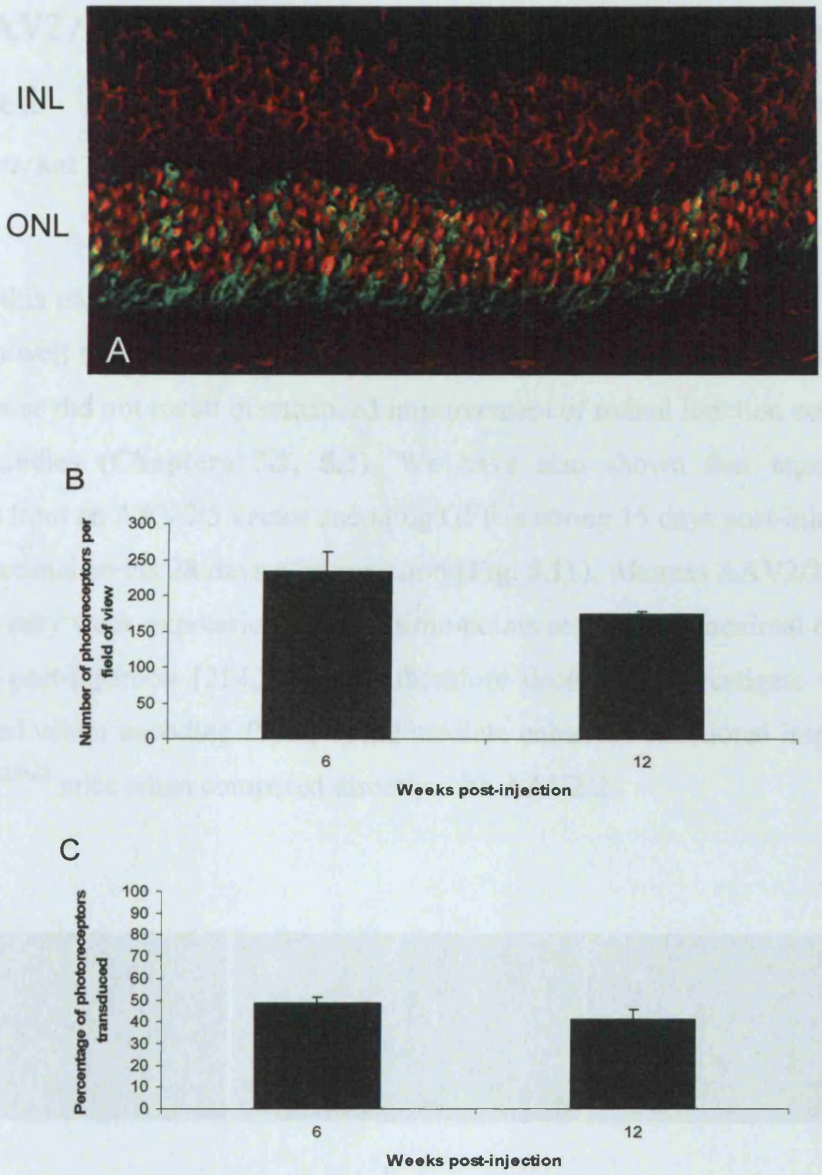


Fig. 5.10: Sub-retinal delivery of AAV.MOPS.Prph2.IRES.GFP does not confer selective advantage to transduced cells in *Prph2*$^{Rd2/Rd2}$ mice; six-weeks post injection, cryosections were taken from treated eyes and confocal images taken at fixed positions in each eye. (A) representative image showing GFP-positive photoreceptors within PI-cunterstained outer nuclear layer. (B) Average number of photoreceptors per field of view at 6 weeks post injection (235.7 ± 27.7) is significantly greater than at 12 weeks post-injection, demonstrating that between the two time-points analysed the outer nuclear layer was degenerating ($p = 0.02$, $n = 4$). (C) As a percentage of total photoreceptor cells, there are fewer transduced cells surviving at 12 weeks post-injection (41.4 % ± 4 %, $p = 0.04$, $n = 4$) than at six weeks post-injection (48.9 % ± 2.6 %), suggesting there is no selective advantage conferred to cells that express *Prph2* compared to non-transduced neighbouring cells.

5.6 AAV2/5-mediated gene replacement therapy results in enhanced *Prph2*-mediated functional improvement in *Prph2*$^{Rd2/Rd2}$ mice

In this chapter we have shown that *Prph2* expression mediated by high-titre AAV2/2 is well tolerated in wild-type mice, but that sub-retinal delivery of this virus in PND7 mice did not result in enhanced improvement of retinal function compared to previous studies (**Chapters 5.2, 5.3**). We have also shown that reporter gene expression from an AAV2/5 vector encoding GFP is strong 15 days post-injection and reaches maximal levels 28 days post-injection (**Fig. 5.11**), whereas AAV2/2 is known to mediate only weak expression at these time-points and to give maximal expression six weeks post-injection [214,265]. We therefore decided to investigate whether a pseudotyped vector encoding *Prph2* could mediate enhanced functional improvement in *Prph2*$^{Rd2/Rd2}$ mice when compared directly with AAV2/2.

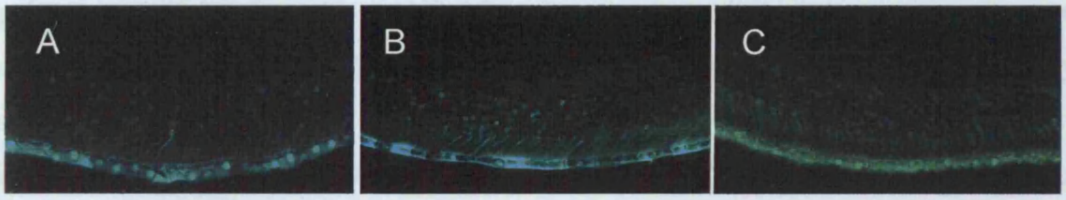


Fig. 5.11: AAV2/5 encoding GFP mediates strong transgene expression two weeks post-injection, earlier than AAV2/2; cryosections taken from wild-type mouse eyes following sub-retinal delivery of AAV2/5.CMV.GFP showing onset of reporter gene expression at 15 days post-injection (A), and strong expression 21 days (B) and 28 days (C) post-injection. Courtesy of Dr. M Rajah.

Using the Sepharose-mucin FPLC protocol described in **Chapter 3.4**, AAV2/5.Rho.Prph2 was produced in order to validate this pseudotyped virus as a gene replacement vector in the *Prph2*$^{Rd2/Rd2}$ mouse. AAV2/2.Rho.Prph2 was produced as previously described [246] as a control virus, and the titre of both AAV2/2- and AAV2/5.Rho.Prph2 was determined on the same dot blot to obtain an accurate measure of the number of genome particles produced; the AAV2/5 titre was shown to be $2x10^{13}$ genomes per ml, and the AAV2 titre was $2x10^{12}$ genomes per ml. In order to assess whether treatment with AAV2/5.Rho.Prph2 would result in enhanced functional rescue compare with AAV2/2.Rho.Prph2, *Prph2*$^{Rd2/Rd2}$ mice (post-natal day 10, n = 8) received sub-retinal injections of these viruses at equal titre (AAV2/5 was diluted 1/10), with right eyes treated with AAV2/5 and left eyes with AAV2/2. Four weeks post-injection (this is the earliest time point at which ERG recordings can be reliably made in young mice) both AAV2.Rho.Prph2-treated eyes (96.2 μV ± 31μV at 100mcds/m^2) and AAV2/5-treated eyes (125.7 μV ± 22μV) showed b-wave amplitudes greater than those expected from untreated eyes (usually between 40 and 60 μV at this age in *Prph2*$^{Rd2/Rd2}$ mice) (**Fig. 5.12**). ERGs were then recorded every two weeks for 16 weeks, at which time point the animals were sacrificed. At all time points eyes treated with AAV2/5.Rho.Prph2 showed higher average b-wave amplitudes than those treated with AAV2.Rho.Prph2; this difference was statistically significant at weeks 8 (p=0.001), 10 (p=0.007), 12 (p=0.02) and 16 (p=0.04) post-injection, and returned to being non-significant (p=0.055) at week 20 (**Fig. 5.12**). This indicates that AAV2/5 is able to mediate significantly enhanced functional improvement in this model of retinal degeneration when compared to titre-matched AAV2 that contains the same expression cassette. Following the final ERG recording 20 weeks post-injection, the mice were sacrificed and processed for histological analysis by resin-embedded sectioning. Sections from AAV2/5-injected and AAV2/2-injected eyes were compared, there did not appear to be a greater number of cells surviving in eyes treated with AAV2/5 (**Fig. 5.13A, B**). We were unable to show intact outer segments persisting at this stage on electron microscopy (**Fig. 5.13C, D**).

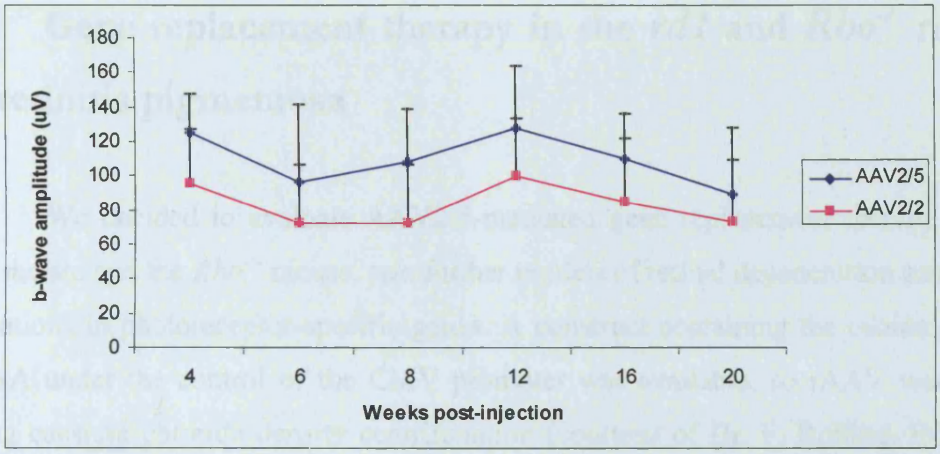


Fig. 5.12: AAV2/5.Rho.Prph2 mediates enhanced improvement in retinal function in *Prph2*$^{Rd2/Rd2}$ mice when compared to AAV2/2.Rho.Prph2; averaged b-wave amplitudes taken after injection of either AAV2/5.Rho.Prph2 (right eye) or AAV2/2.Rho.Prph2 (left eye) show that AAV2/5 results in higher amplitudes at all time points assessed.

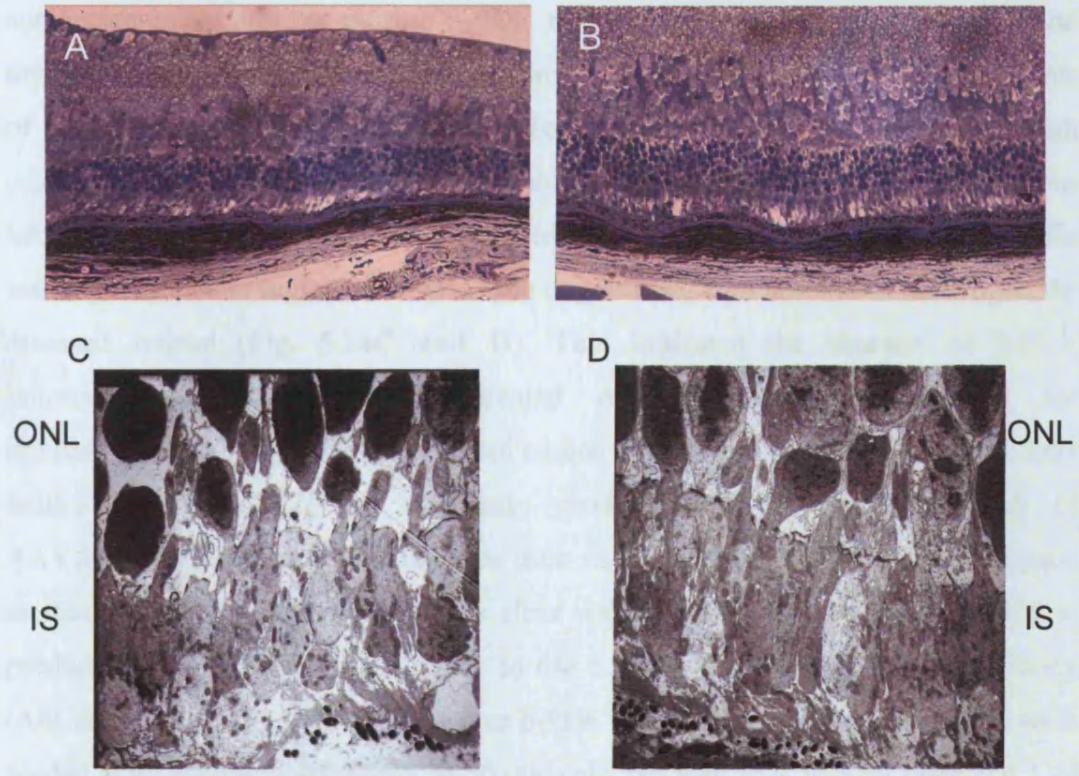


Fig. 5.13: AAV2/5.Rho.Prph2 does not appear to delay photoreceptor cell loss when compared to AAV2/2.Rho.Prph2; at five months post-injection, there are no more photoreceptors surviving in AAV2/5-treated eyes (B) compared with AAV2/2-treated eyes (A) as seen on semi-thin resin-embedded histological section. We were unable to show persistence of organised outer segments at this time-point upon electron microscopy in eyes treated with AAV2/2 (C) or AAV2/5 (D).

5.7 Gene replacement therapy in the *rd1* and *Rho*$^{-/-}$ models of retinitis pigmentosa

We decided to evaluate AAV2/5-mediated gene replacement therapy in the *rd1* mouse and the *Rho*$^{-/-}$ mouse, two further models of retinal degeneration caused by mutations in photoreceptor-specific genes. A construct containing the canine *β-PDE* cDNA under the control of the CMV promoter was available, so rAAV was made using caesium chloride density centrifugation (courtesy of Dr. F. Rolling, INSERM Nantes, France), as previously described [268]. This virus was evaluated *in vivo* as a gene replacement vector. PND1 *rd1* mice (on a C3H genetic background) received double sub-retinal injections of 1 µl AAV2/5.CMV.βPDE virus suspension ($5x10^{11}$ genomes/ml). Five and seven days post-injection, mice were sacrificed and their eyes processed for cryosectioning. Immunohistochemistry on 10 µm sections using a rabbit anti-serum raised against murine β-PDE revealed no staining in both treated and untreated eyes, whereas wild-type mice showed diffuse staining in the outer segments of photoreceptors (**Fig. 5.14A and B**). To ascertain whether the rabbit anti-serum cross-reacts with canine β-PDE, immunohistochemistry was performed on sections taken from wild-type and *rcd1* dogs. This revealed faint but apparently specific staining in the outer segments of wild-type dog retinae, with absence of staining in the diseased retinae (**Fig. 5.14C and D**). This indicated the absence of β-PDE immunostaining in the AAV2/5-treated *rd1* eyes was not due to the immunohistochemistry failing to detect canine β-PDE protein, but due to a likely failure of the AAV2/5 to efficiently produce transgene. A new batch of AAV2/5.CMV.βPDE was isolated, this time using the sepharose-mucin purification method described in **Chapter 3**. This virus was evaluated *in vitro* for its ability to produce β-PDE protein; we were able to use a newly available polyclonal antibody (AbCam, Cambridge, UK) raised against β-PDE to detect expression. 293T cells were seeded at an approximate density of 50,000 cells per well in a 24-well plate and 1 µl AAV2/5.CMV.βPDE ($2x10^{12}$ genomes/ml) was added in triplicate. As controls, wells were transfected with pD10.CMV.βPDE, to verify whether the expression cassette itself was able to produce mature protein *in vitro*.

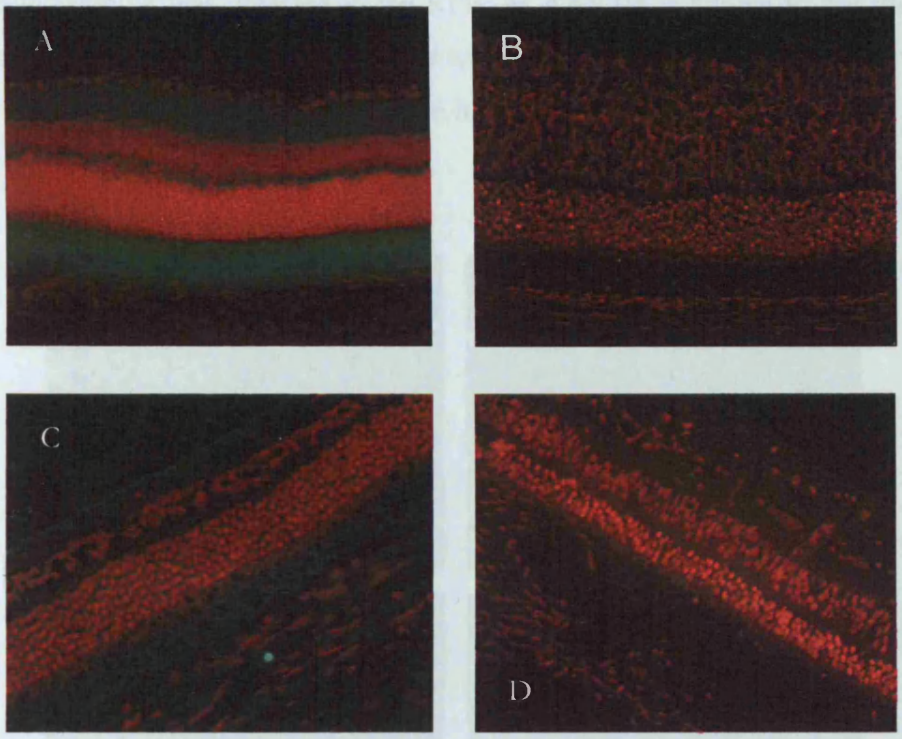


Fig. 5.14 AAV2/5-mediated gene replacement therapy in *rd1* mouse does not result in detectable β-PDE protein production; immunohistochenistry performed on frozen sections taken from (A) wild-type mouse (diffuse green staining in outer segments is β-PDE); (B) *rd1* mouse treated with AAV2/5.CMV.βPDE at P1 and sacrificed at P11 (no immunostaining is seen in any retinal layers); (C) wild-type dog section (faint punctuate green immunofluorescence corresponds to β-PDE; (D) *rcd1* dog, in which absence of green staining seen in (C) indicates that the antibody used cross-reacts with canine β-PDE protein.

Infection of 293T cells using AAV2/5.CMV.βPDE did not result in detectable levels of β-PDE protein, whereas plasmid transfection using pD10.CMV.βPDE (the plasmid containing AAV2 ITRs that was used to make rAAV) does result in specific βPDE staining, indicating that the expression cassette is able to drive production of βPDE protein (**Fig. 5.15A-C**). The βPDE cDNA was also sequenced, which confirmed that the full-length coding sequence had been cloned into the pD10 plasmid. AAV.CMV.hrGFP was also infected into 293T cells at the same time, to confirm that *in vitro* infection of virus leads to transgene expression (**Fig. 5.15D**). Because the sequence of the cDNA was correct, the plasmid construct was shown to give transgene expression and the titre of the rAAV was high ($5x10^{12}$ genomes/ml), we decided to administer the AAV.CMV.βPDE by sub-retinal injection to P1 *rd1* mice, to evaluate its efficacy in this model of RP. As seen previously, no βPDE expression was seen on cryosections of *rd1*mice sacrificed ten days post-injection. Interestingly,

no immunostaining was detected in the RPE; as AAV2/5 is known to transduce the RPE, and a ubiquitous promoter sequence was used in the expression cassette, we had expected to see some transgene expression in the RPE.

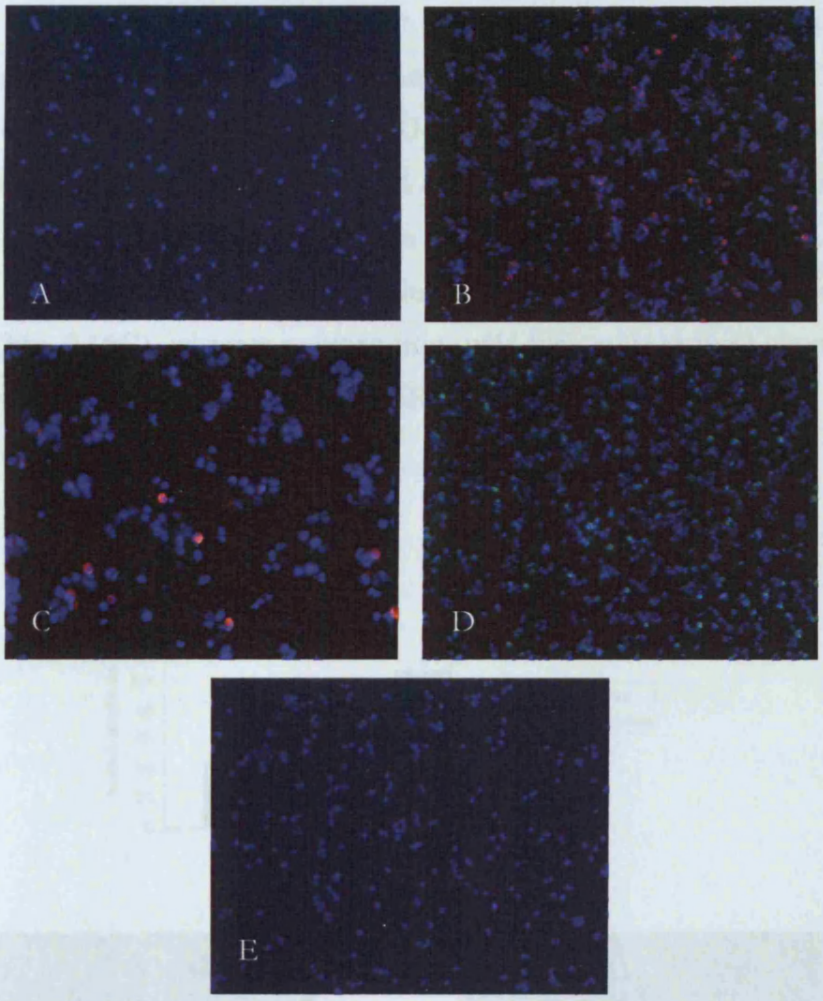


Fig. 5.15: Infection of 293T cells with AAV2/5.CMV.βPDE does not result in detectable β-PDE protein production *in vitro*, whereas plasmid transfection with pD10.CMV.βPDE does; *in vitro* immunocytochemistry performed on 293T cells following (A) infection with AAV2/5.CMV.βPDE; (B, C) plasmid transfection with pD10.CMV.βPDE; (D) infection with AAV2/5.CMV.hrGFP; (E) no treatment. (A) shows no cells stained with anti-β-PDE antibody, only DAPI counter-stained nuclei (blue). (B) shows β-PDE staining in cell bodies of approximately 20% of cells following plasmid transfection; (C) is higher-magnification view of cells in (B). (D) shows that AAV2/5 is able to efficiently infect 293T cells, with hrGFP fluorescence (green) in approximately 50% of cells. All cells counterstained with DAPI, images taken at 20X magnification except (C), which is at 40X.

We also attempted to rescue the *Rho*$^{-/-}$ mouse by using sub-retinal delivery of AAV2/5 encoding the human *Rhodopsin* cDNA. The cDNA was obtained as a kind gift from P. Humphries (Trinity College, Dublin, Ireland), and was sub-cloned into our AAV2 ITR plasmid pD10 to give AAV.boRho.huRho, in which the human *Rhodopsin* gene is driven by the same bovine rhodopsin promoter that is used in our AAV.Rho.Prph2 construct (sub-cloning carried out by Dr. A. MacNeil). rAAV was produced and purified using the sepharose-mucin chromatography method described in **Chapter 3**. Mice were treated at PND 10, and ERG recordings taken 10 days post-injection showed that at 100 mcds/m^2, both treated and untreated eyes had negligible b-wave amplitudes characteristic of the *rhodopsin* knock-out mouse (**Fig. 5.16A**). We were unable to detect rhodopsin immuno-staining in treated eyes sacrificed 21 days post-injection (**Fig. 5.16C**), whereas sections from wild-type mice showed strong staining in photoreceptor outer segments (**Fig. 5.16B**).

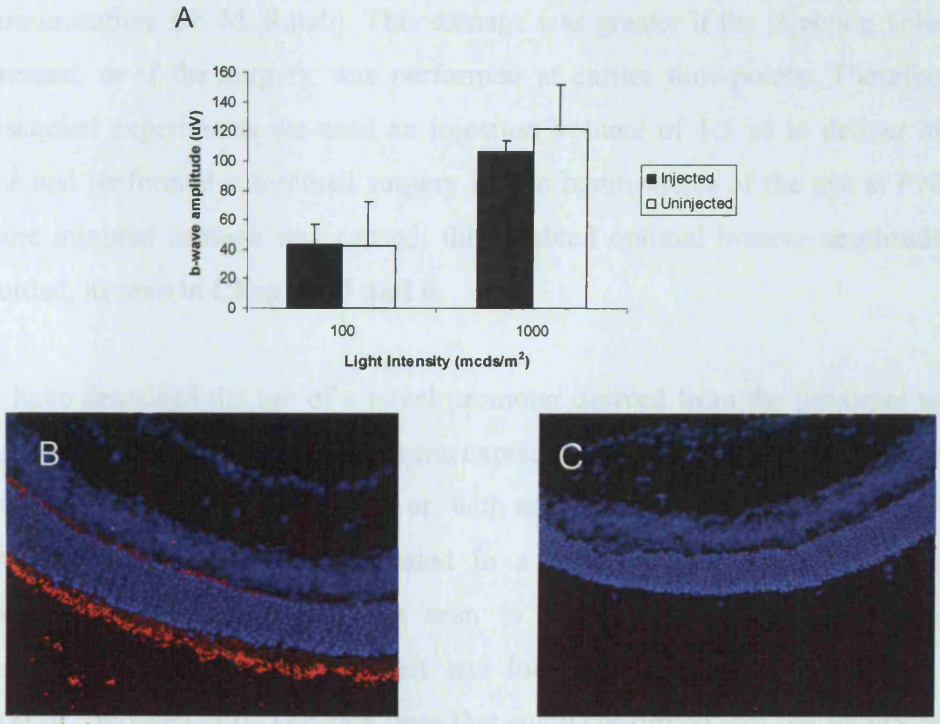


Fig. 5.16: Sub-retinal injection of AAV.boRho.huRho does not result in improved retinal function as assessed by ERG; (**A**) ten days post-injection, ERG recordings taken from *Rho*$^{-/-}$ mice treated with AAV.boRho.huRho showed no improvement in b-wave amplitudes in treated eyes when compared with untreated contralateral eyes. At 1000 mcds/m^2 there are cone-derived b-waves recorded from both eyes, but treated eyes do not have greater amplitudes than untreated eyes. (**B**) Whereas wild-type eyes show strong staining in the inner/outer segments for Rhodopsin (red), *Rho*$^{-/-}$ eyes treated with AAV.boRho.huRho show no Rhodopsin staining.

4.8 Discussion

Having established that high-titre AAV-mediated *Prph2* expression does not significantly reduce retinal function as assayed by ERG, we used several approaches to improve the efficiency of *Prph2* gene delivery to the $Prph2^{Rd2/Rd2}$ retina. The first approach was to use these higher-titre viral preparations, and to perform sub-retinal injections at earlier time-points than previously attempted to initiate therapeutic gene expression earlier. However, there was no additional benefit of this strategy in terms of improved ERG b-waves; the extra trauma of surgical intervention at an earlier age seems to negate the benefit of having an earlier onset of gene expression. This finding is corroborated by data from experiments investigating the consequences of sub-retinal injections in both neonatal and adult mice; it was determined that injection of more than 1.5 µl PBS in PND10 mice caused significant damage to retinal function as assessed by ERG, as did repeated injections at PND 10 and PND 15 (personal communication, Dr. M. Rajah). This damage was greater if the injection volume was increased, or if the surgery was performed at earlier time-points. Therefore in all subsequent experiments we used an injection volume of 1.5 µl to deliver high-titre virus and performed sub-retinal surgery in two hemispheres of the eye at PND 10 to ensure minimal damage was caused; this enabled optimal b-wave amplitudes to be recorded, as seen in **Chapters 5 and 6**.

We have described the use of a novel promoter derived from the proximal region of the mouse opsin gene to drive transgene expression in photoreceptors. This expression is restricted to the outer nuclear layer, with no RPE expression detected. The MOPS promoter was subsequently evaluated in a gene replacement strategy in which efficient *RPGRIP* expression was seen in rods that lead to restoration of the connecting cilium trafficking defect and long-term improvement in ERG in the $RPGRIP^{-/-}$ mouse [239]. The data from that study confirmed that the MOPS promoter drives strong transgene expression in rod cells. The lack of transgene expression in cone cells that is described above appears to contradict a previous report suggesting that a similar fragment of the mouse opsin promoter does drive expression in cones [282]. However this study differed from ours in certain respects. Firstly, we used a different fragment of the proximal region of the mouse *rhodopsin* gene (nucleotides -

218 to +17 relative to start codon) than the authors of this study used (-385 to +86). It is difficult to say whether this affected the ability of our promoter to mediate transgene expression in cone cells. In addition our method of analysing cone-specific gene expression was also different; whereas it appears that Glushakova *et al* used standard fluorescent microscopy and a digital camera for imaging their cryosections, we used a confocal scanning laser microscope, which provides greater resolution within single optical slices of 1 μm in thickness. This technique allows greater certainty in co-localisation studies, meaning that whilst on a fluorescent micrograph a cell may appear to be expressing both PNA and GFP, the two fluorescent signals may originate in two different cells that lie adjacent to or above one another. We therefore report that, using confocal imaging, we were unable to see any PNA-positive cone cells that co-expressed GFP, whereas upon light microscopy some cells did appear to express both markers.

By investigating the effect of transduction with a *Prph2* cDNA on the survival of photoreceptors over time, our findings suggest that there is no selective advantage conferred to transduced rod cells when compared with untransduced neighbouring cells. Indeed, there may be a small effect to the contrary, as it appears that 7.5 % fewer surviving cells are transduced at 12 weeks post-injection than at 6 weeks post-injection. Although it is not likely that this small reduction in survival of transduced cells is due to a specific toxic effect of *Prph2* cDNA expression, it does warrant further investigation. It may be that the GFP expression driven by our MOPS.Prph2.IRES.GFP construct causes some toxicity, as has been seen with high-level GFP expression in the past [283].

We have also shown that AAV2/5 mediates efficient gene replacement therapy in $Prph2^{Rd2/Rd2}$ mice, which results in significantly better ERG b-wave amplitudes than seen with AAV2. This result validates the use of AAV2/5-mediated gene replacement for photoreceptor-specific disorders, although there does not appear to be any effect on the rate of photoreceptor cell loss; this is discussed in more detail in **Chapter 7**. Despite the enhanced functional rescue seen in this model following treatment with AAV2/5.Rho.Prph2, using pseudotyped AAV does not appear to result in efficient gene replacement therapy in the *rd1* mouse or the $Rho^{-/-}$ mouse. We have shown that the expression cassette drives efficient gene expression following *in vitro* plasmid

transfection, but that *in vivo* we see no photoreceptor cells expressing β-PDE following administration of virus. It is therefore not possible to conclude whether the *rd1* mouse can be effectively treated using a pseudotyped AAV vector, and this warrants further investigation as discussed in **Chapter 6**. Similarly, the apparent failure of AAV2/5.boRho.huRho to mediate efficient transgene expression in the *rhodopsin* knock-out mouse warrants further investigation; although the sequence of both the promoter element in the construct and the cDNA have been verified, it is not clear whether the rAAV drives expression of *Rhodopsin in vitro*. The use of a photoreceptor-specific promoter makes it technically challenging to assess this construct *in vitro*, as we are unable to reliably culture photoreceptor-derived cell lines. Cell lines such as Y79 cells have been described [47], which if cultured successfully would allow *in vitro* testing of constructs driven by photoreceptor-specific promoters such as MOPS and Rho.

6 Neuroprotection in combination with gene replacement therapy in rodent models of RP

6.1 Introduction

As discussed in **Chapter 1.14**, many gene replacement strategies provide only transient improvement in retinal function, and, with a few notable exceptions are unable to delay or prevent the apoptotic loss of photoreceptors. With delivery of recombinant growth factors failing largely due to their very short ocular half-lives, viral vectors have been used to deliver genes encoding neurotrophic factors to delay apoptosis in retinal degeneration. However, of major concern is the deleterious effect on retinal function, both in models of retinitis pigmentosa and in wild-type rodents, that results from AAV-mediated *CNTF* delivery [253-255]. The aim of the experiments in this chapter is to investigate whether the reduction in ERG b-wave mediated by CNTF is dependent on the dose administered, and if so whether a dose can be identified that does not reduce normal retinal function but that enhances gene replacement therapy by delaying photoreceptor loss. In addition, the long-term consequences of *CNTF* gene delivery are investigated in a model of retinal degeneration in which retinal function and photoreceptor numbers decline gradually over a period of one year.

Glial cell line-derived neurotrophic factor has shown much promise in gene therapy applications in the CNS, but is yet to be extensively studied as a neuroprotective agent in retinal degeneration. Therefore we have evaluated GDNF in terms of its effect on normal retinal function, in order to determine whether GDNF exerts similar deleterious effects to CNTF. We have also evaluated the efficacy of AAV-mediated *GDNF* expression to enhance gene replacement therapy in two rodent models of retinitis pigmentosa, in order to assess whether GDNF is able to enhance the morphological and electrophysiological improvements seen following gene replacement.

6.2 Deleterious effects of AAV-mediated *CNTF* expression are dose-dependent

In order to investigate whether the detrimental side effects of AAV-mediated CNTF expression are dose-dependent, we carried out a dose response experiment *in vitro* and *in vivo*. To determine whether a reduction in particle titre of AAV.CMV.CNTF results in a reduction of CNTF production, a sandwich ELISA was performed to determine the level of CNTF protein produced. 50,000 293T cells were seeded per well of a 24-well plate, and $5x10^8$, $5x10^7$ and $5x10^6$ particles of AAV.CMV.CNTF were added to the wells in triplicate. The amount of CNTF protein present in the supernatant was dependent on the dose of virus (**Fig. 6.1**); the cells infected with $5x10^8$ particles gave the highest level of CNTF protein (20.6 ng/ml ±1 ng/ml), with a 1/10 dilution giving an intermediate amount (8 ng/ml ±2.1 ng/ml) and the 1/100 dose resulting in the lowest amount of CNTF protein produced (3.4 ng/ml ±0.4 ng/ml). We injected three groups of two month-old wild-type CBA mice with a dilution series of AAV.CMV.CNTF virus; ($1x10^{10}$ genomes/ml, as used previously [255], and 10- and 100-fold dilutions of this stock). Right eyes were injected in superior and inferior hemispheres with 2 µl of the appropriate dilution, with left eyes serving as uninjected controls. Six weeks and two months post-injection, pan-retinal function was assessed by ERG. A severe deleterious effect on retinal function was observed in the group of mice injected with the highest concentration of virus (**Fig. 6.2**); injected right eyes showed a 40 % reduction in ERG b-wave amplitude as compared with uninjected contralateral control eyes ($p=0.01$). Mice injected with a 1/10 dilution of the neat virus showed a smaller decrease in b-wave amplitude (24 %), whilst the group of mice that received a 1/100 dilution showed no significant change in ERG. This clearly demonstrates the dose-dependent relationship between AAV-mediated *CNTF* expression and reduction in retinal function.

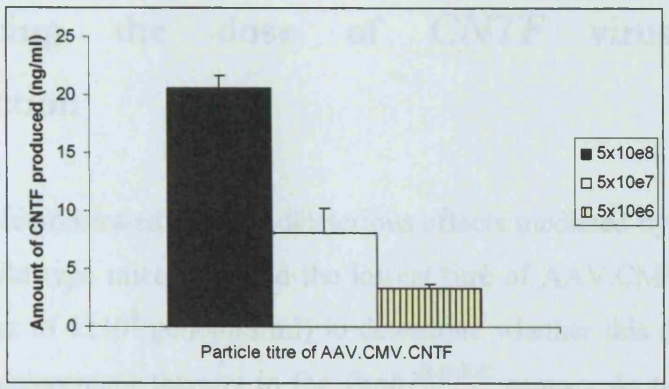


Fig. 6.1: Amount of CNTF protein produced *in vitro* following infection with AAV.CMV.CNTF is dose-dependent; 50,000 293T cells were infected with $5x10^8$, $5x10^7$ or $5x10^6$ virus particles, and CNTF production assessed by sandwich ELISA. CNTF was significantly higher in cells that received the highest dose compared with the 1/10 dilution ($p<0.02$) and with the 1/100 dilution ($p<0.01$).

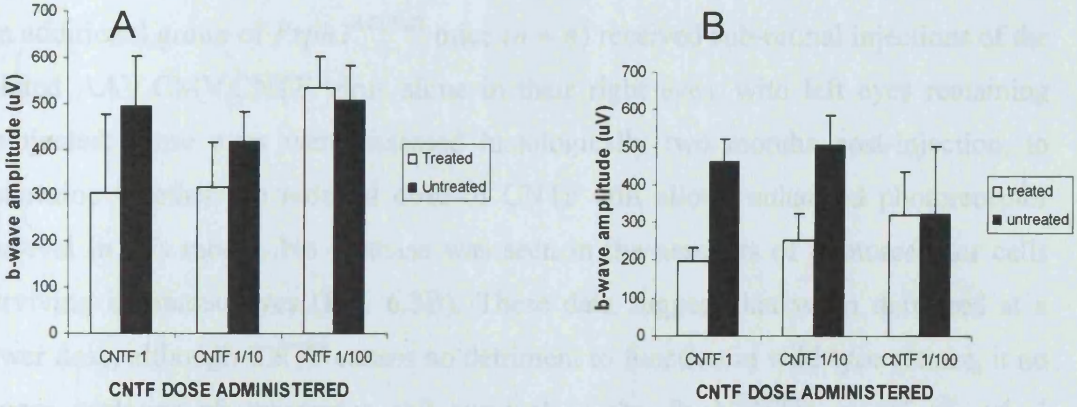


Fig. 6.2: Reduction in retinal function following AAV-mediated *CNTF* delivery is dose-dependent; In wild-type mice sub-retinal injection of AAV.CMV.CNTF at the highest dose (CNTF 1, $5x10^{10}$ particles per ml) resulted in a large reduction in ERG b-wave amplitude (40 %, $n = 4$, $p = 0.01$) in treated eyes when compared with untreated eyes. A ten-fold dilution of the virus (CNTF 1/10) resulted in an intermediate level of reduction in function (24 %, $n = 4$, $p = 0.009$), and a 100-fold dilution (CNTF 1/100, $n = 4$) resulted in no significant reduction in function. ERG recordings taken at (A) six weeks and (B) eight weeks post-injection.

6.3 Reducing the dose of *CNTF* virus precludes neuroprotection

Having demonstrated that the deleterious effects mediated by CNTF are dose dependent in wild type mice, we used the lowest titre of AAV.CMV.CNTF (CNTF 1/100, equivalent to $1x10^8$ genomes/ml) to determine whether this dose was able to enhance gene replacement therapy in the $Prph2^{Rd2/Rd2}$ mouse. At post-natal day 10 $Prph2^{Rd2/Rd2}$ mice received double sub-retinal injections of a combination of the diluted AAV.CMV.CNTF virus and AAV.Rho.Prph2 in the right eye, with left eyes receiving AAV.Rho.Prph2 only (n = 6, total volume injected per hemisphere 1.5 µl). No significant increase in b-wave amplitude was noted in eyes treated with a combination of AAV.CMV.CNTF and AAV.Rho.Prph2 over eyes treated with gene replacement only (**Fig. 6.3A**). This suggests that treatment with the reduced dose of CNTF does not result in enhanced function when compared with gene replacement alone.

An additional group of $Prph2^{Rd2/Rd2}$ mice (n = 4) received sub-retinal injections of the diluted AAV.CMV.CNTF virus alone in their right eyes, with left eyes remaining uninjected; these eyes were assessed histologically two months post-injection, to determine whether the reduced dose of CNTF still allows enhanced photoreceptor survival in this model. No increase was seen in the numbers of photoreceptor cells surviving in treated eyes (**Fig. 6.3B**). These data suggest that when delivered at a lower dose, although CNTF causes no detriment to function in wild-type retinae, it no longer prolongs photoreceptor cell survival in the $Prph2^{Rd2/Rd2}$ model of retinal degeneration.

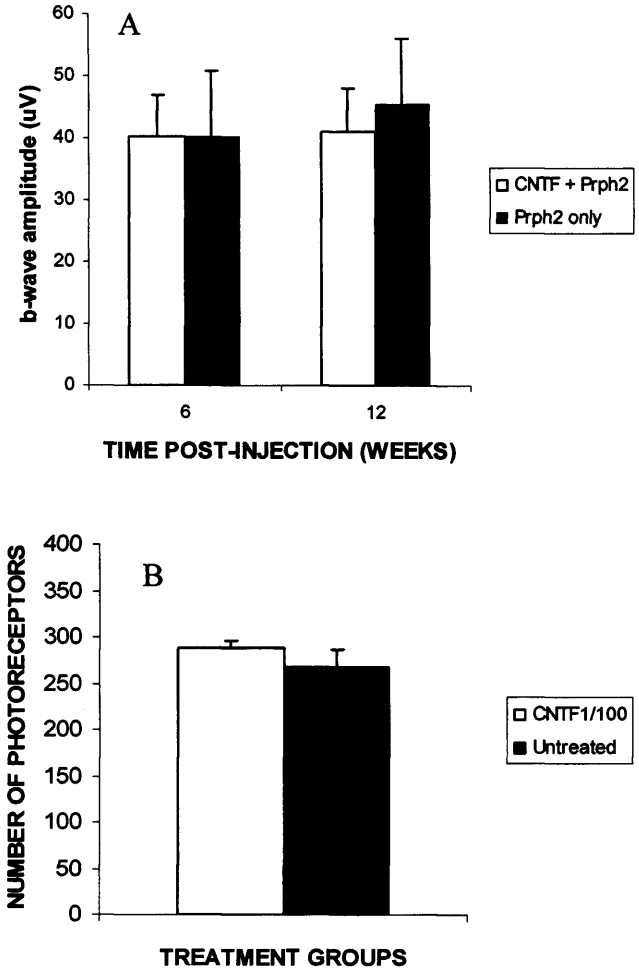


Fig. 6.3: Reducing CNTF dose precludes neuroprotection in *Prph2*$^{Rd2/Rd2}$ mouse; (A) there is no significant difference in ERG b-wave amplitudes in *Prph2*$^{Rd2/Rd2}$ mice following co-injection of AAV.CMV.CNTF (CNTF 1/100) and AAV.Rho.Prph2, when compared with injection of AAV.Rho.Prph2 alone (n = 4). (B) There is no significant difference in the numbers of surviving photoreceptors in *Prph2*$^{Rd2/Rd2}$ mice following injection of diluted AAV.CMV.CNTF (CNTF 1/100) when compared with uninjected control eyes. Photoreceptor numbers were quantified by taking photomicrographs of sections at fixed positions from a serially sectioned orientated eye.

6.4 Long-term *CNTF* expression in *Prph2*$^{Rd2/+}$ mice results in reduced function and photoreceptor damage

CNTF gene delivery that prevents photoreceptor death also results in a diminished ERG, but if this is stably maintained until beyond the point at which untreated eyes have no function, RP patients may benefit from CNTF-mediated photoreceptor survival. In order to determine whether this might be the case, we evaluated the effects of AAV-mediated *CNTF* expression in *Prph2*$^{Rd2/+}$ mice, a murine model of RP with a slow rate of degeneration. In *Prph2*$^{Rd2/+}$ mice, photoreceptors are lost gradually, with the ERG declining from near wild-type levels to around 50 % thereof at one year of age [284]. We treated *Prph2*$^{Rd2/+}$ mice with intravitreal injection of AAV.CMV.CNTF (at a titre of $1x10^{10}$ genomes/ml, which has been shown to prevent cell death whilst reducing retinal function) and followed the animals' retinal function, as assayed by ERG, over nine months. Five months post-injection the damage to retinal function was apparent; ERG traces were markedly reduced in CNTF-treated eyes as compared with uninjected internal control eyes and eyes that had received intravitreal injection of AAV.CMV.GFP as a control (**Fig. 6.4A**). Rather than observing a diminished ERG that is stably maintained, we observed that retinal function in CNTF-treated eyes became progressively worse over time with decreased b-wave amplitudes even at long-term time-points five and nine months post-injection (**Fig. 6.4B**).

Twelve months post-injection the mice were sacrificed and enucleated, and the eyes were processed as follows: after eyecup dissection where the lens and cornea were removed, half of the neurosensory retina was processed for cryosections and half for resin-embedded semi- and ultra-thin sections. Immunohistochemistry performed on cryosections (see **Chapter 2.5.3** and **Appendix I**) showed that eyes treated with AAV.CMV.CNTF had greatly reduced levels of cone opsin staining (**Fig. 6.5B, C**) when compared with untreated eyes (**Fig. 6.5A**); some sections taken from CNTF-treated eyes contained one or two opsin-positive cells only (**Fig. 6.5B**), whereas most sections had no cone opsin immunopositivity at all (**Fig. 6.5C**). Even where there was cone opsin staining in CNTF-treated eyes, the cone opsin was often mis-localised in the inner segment and the cell bodies, an example of which is shown in **Fig. 6.5D**.

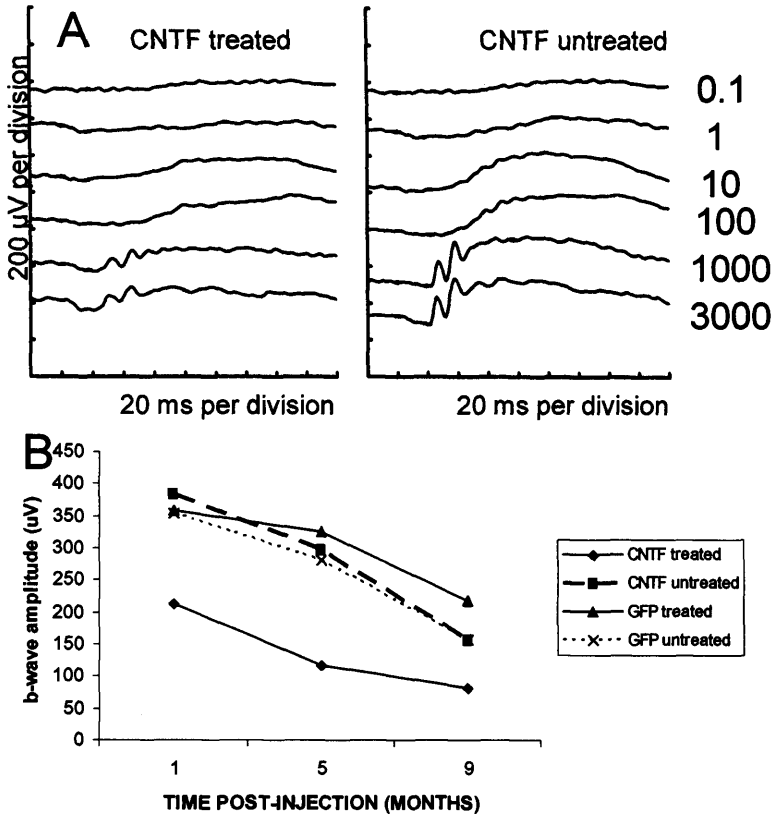


Fig. 6.4: AAV-mediated *CNTF* expression reduces retinal function in *Prpb2*$^{Rd2/+}$ mice; (A) ERG intensity series, from a *Prpb2*$^{Rd2/+}$ mouse five months after intravitreal injection of AAV.CMV.CNTF (1 x 10^{10} genomes/ml) in one eye. There is a decline in function in the injected eye (left panel) when compared with the uninjected contralateral eye (right panel). Light intensity shown to the right of each trace in mcds/m^2. (B) b-wave amplitudes from *Prpb2*$^{Rd2/+}$ mice following monocular intravitreal injection of either AAV.CMV.CNTF or AAV.CMV.GFP (6 animals in each group). At one month post-injection, CNTF treated eyes have lower b-wave amplitudes than their uninjected contralateral eyes (CNTF untreated), as well as GFP treated eyes and their uninjected contralateral eyes (GFP untreated). These reduced amplitudes continue to deteriorate and remain lower than controls.

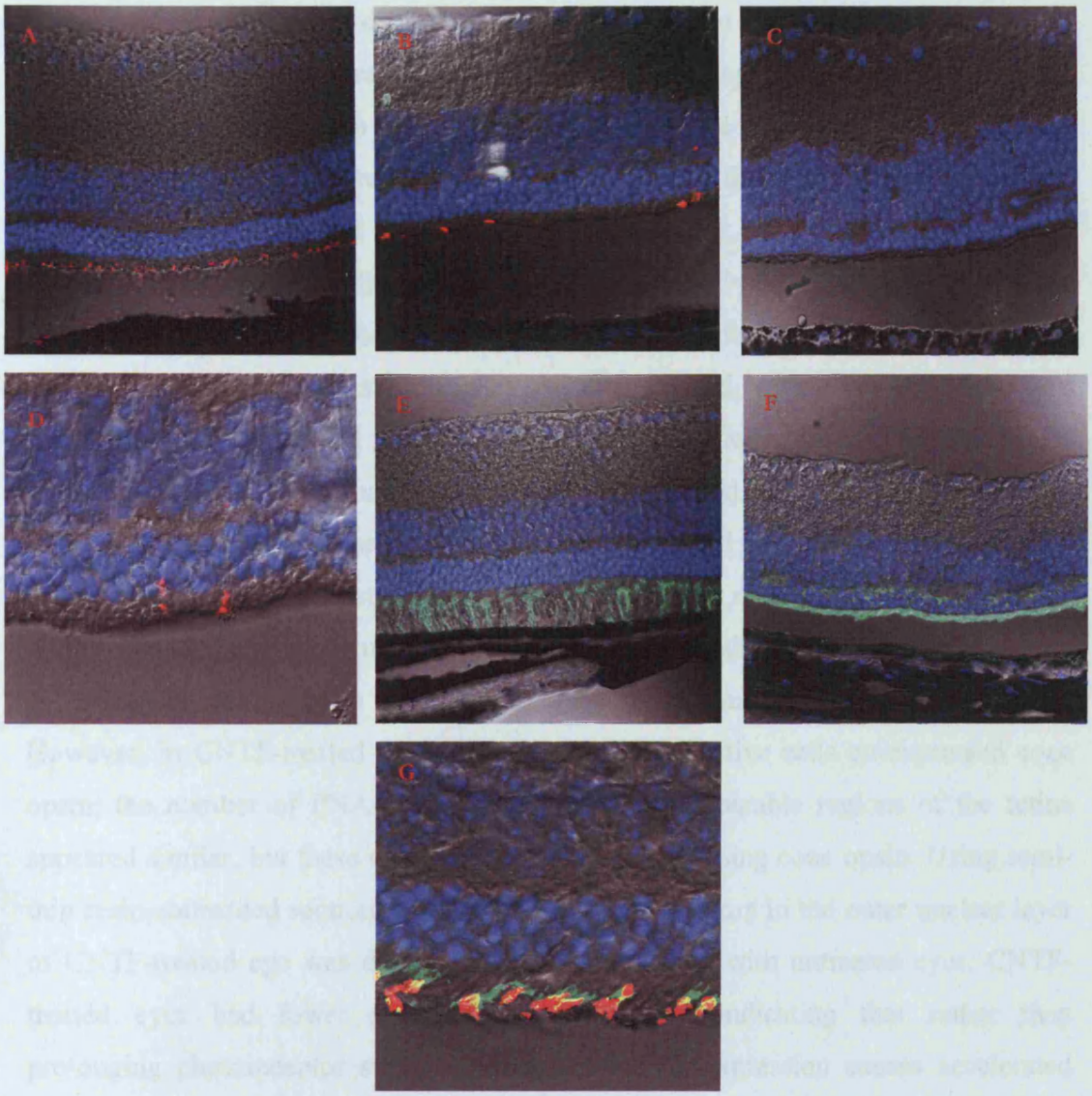


Fig. 6.5: AAV-mediated *CNTF* expression causes loss of cone opsin expression but not loss of cone cells in *Prph2*$^{Rd2/+}$ mice; (A) Cone opsin staining in untreated *Prph2*$^{Rd2/+}$ eye at 15 months of age. Some sections from *Prph2*$^{Rd2/+}$ eyes treated with AAV.CMV.CNTF have small numbers of cone opsin-positive photoreceptors (B), but most sections show no cone opsin staining at all (C). Where cone opsin is present in CNTF-treated eyes it is mislocalised to the inner segment and cell body (D). (E) Peanut agglutinin (PNA) staining in untreated *Prph2*$^{Rd2/+}$ eye (green) that localises to the inner and outer segment sheaths. PNA staining in CNTF-treated eyes is disorganised and some staining is seen in the outer nuclear layer (F). PNA and cone opsin staining can be co-localised in untreated *Prph2*$^{Rd2/+}$ eyes (G), but not in CNTF-treated eyes.

In addition, the outer nuclear layer in CNTF-treated eyes appeared thinner and contained fewer photoreceptor nuclei, which was subsequently confirmed using resin-embedded sections (see below). To determine whether the reduction in cone opsin staining was due to loss of cone photoreceptors, or due to cones no longer expressing opsin despite surviving, peanut agglutinin (PNA) staining was combined with cone opsin staining. PNA is a lectin molecule that binds specifically to galactose-galactosamie disaccharide residues found on the sheaths that surround cone inner and outer segments [281], and as such is used to stain for cone photoreceptors. PNA staining was found to be largely normal in untreated *Prph2*$^{Rd2/+}$ retinae, with cone inner- and outer segments being clearly demarcated by this lectin (**Fig. 6.5E**). CNTF-treated eyes showed PNA staining that was disorganised, with no defined inner and outer segments visible and mis-localised in the outer nuclear layer (**Fig. 6.5F**). The inner and outer segments may have appeared disorganised because the photoreceptors in the CNTF-treated eyes seem to be at a more advanced stage of degeneration, with more cells having been lost. However, we were able to detect cone opsin and PNA double stained cells in the untreated eyes (**Fig. 6.5G**), indicating that cone cells were surviving and continued to express cone opsin at this time point in *Prph2*$^{Rd2/+}$ mice. However, in CNTF-treated eyes, virtually no PNA-positive cells co-expressed cone opsin; the number of PNA-positive cone cells in comparable regions of the retina appeared similar, but these cones were no longer expressing cone opsin. Using semi-thin resin-embedded sections the number of photoreceptors in the outer nuclear layer of CNTF-treated eye was determined; when compared with untreated eyes, CNTF-treated eyes had fewer photoreceptors surviving, indicating that rather than prolonging photoreceptor survival, long-term *CNTF* expression causes accelerated degeneration in this model of RP.

6.5 AAV-mediated *GDNF* expression enhances retinal function in combination with gene replacement in *Prph2*$^{Rd2/Rd2}$ mouse and RCS rat

In order to assess the potential of GDNF as a neuroprotective agent, we constructed an AAV vector containing a mouse *GDNF* cDNA under the control of a CBA promoter. The *GDNF* gene was available in an AAV2 plasmid downstream of a CMV promoter; however, there was also another CMV sequence and the eGFP gene in the same expression cassette. This repetition of the CMV promoter within the expression cassette meant the pD10.CMV.GDNF.CMV.GFP plasmid was highly prone to recombination, and previous attempts to produce rAAV using this construct resulted in infectious virus that expressed GFP *in vitro*, but GDNF protein production was below the detection limit of the sandwich ELISA used to assess GDNF levels (data not shown). The *GDNF* cDNA was therefore sub-cloned from this plasmid by digestion with *SphI*, followed by Vent polymerase end-filling, and *NotI* digestion; this gave a 1400 bp fragment consisting of the proximal CMV immediate early enhancer and the *GDNF* cDNA. This was ligated to a 7.3 kb fragment derived from pD10.CBA.IGF1.IRES.GFP, that had also been digested using the same enzymes, in place of the *IGF1* cDNA. As the CBA promoter fragment is made up of the proximal CMV enhancer sequence and the promoter region from the chicken *β-actin* gene, ligating the two fragments together reconstituted the full-length CBA promoter (**Fig. 6.6**). This new plasmid, pD10.CBA.GDNF.IRES.GFP, was used to make recombinant AAV, and this was assessed *in vitro* for its ability to produce GDNF protein. An ELISA was performed on supernatants collected from 293T cells infected with AAV.CBA.GDNF; supernatants from uninfected cells and cells infected with an unrelated virus (AAV.CMV.IL10) were used as control samples. Both control groups showed no detectable GDNF production above the lower detection limit of the assay (8 pg/ml). In contrast, cells infected with $2x10^9$ particles of AAV.CBA.GDNF gave 3074 pg/ml (± 130 pg/ml), and those infected with $2x10^7$ particles gave a lower level of GDNF protein (1012 pg/ml, ± 146 pg/ml). This showed the vector is effective at producing high levels of GDNF protein.

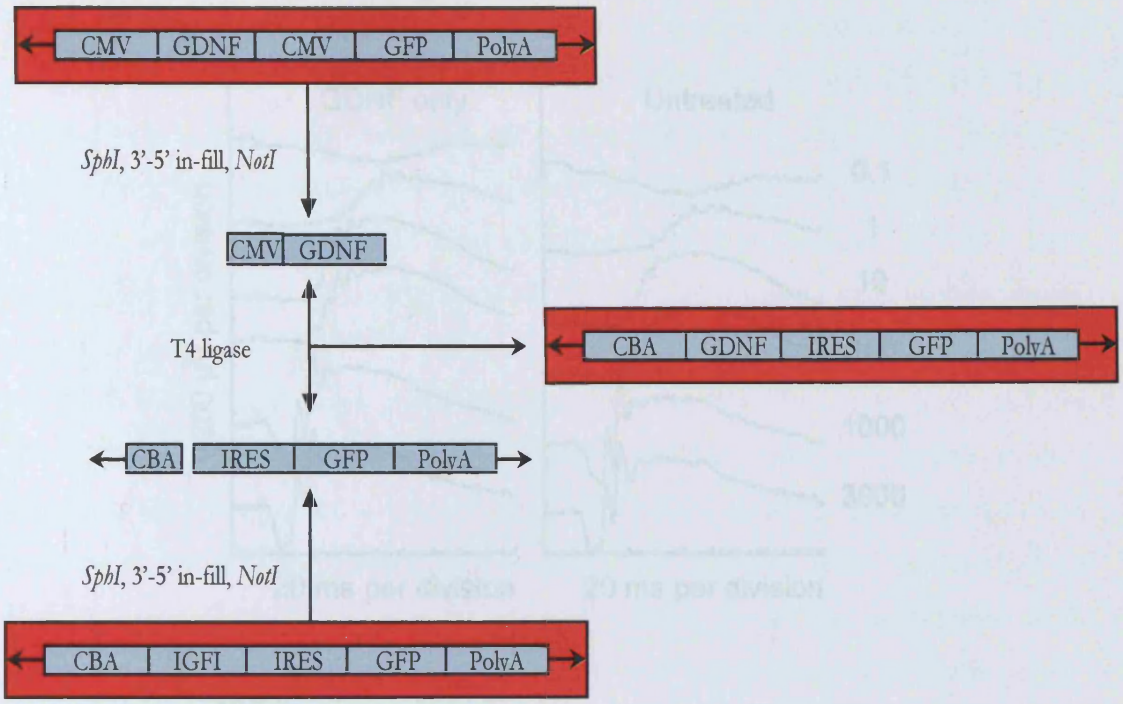


Fig. 6.6: Sub-cloning strategy used to generate AAV plasmid containing *GDNF* cDNA upstream of IRES.GFP sequence; the *GDNF* cDNA was excised from pD10.CMV.GDNF.CMV.GFP along with part of the CMV promoter, and ligated to a fragment of pD10.CBA.IGFI.IRES.GFP, which reconstituted the full-length CBA promoter and gave pD10.CBA.GDNF.IRES.GFP.

To assess the impact of this vector on normal retinal function *in vivo*, wild-type C57/Bl6 mice received sub-retinal injections of AAV.CBA.GDNF ($2x10^{12}$ particles/ml) in the right eye (2 µl), with left eyes left untreated. ERG recordings from dark-adapted mice were taken eight weeks post-injection. AAV-mediated *GDNF* expression did not affect normal retinal function as assayed by electroretinography **(Figs. 6.7A and B)**; GDNF-treated eyes had ERGs with normal waveform and a- and b-wave amplitudes, with no statistically significant reduction in average b-wave following AAV-mediated *GDNF* expression.

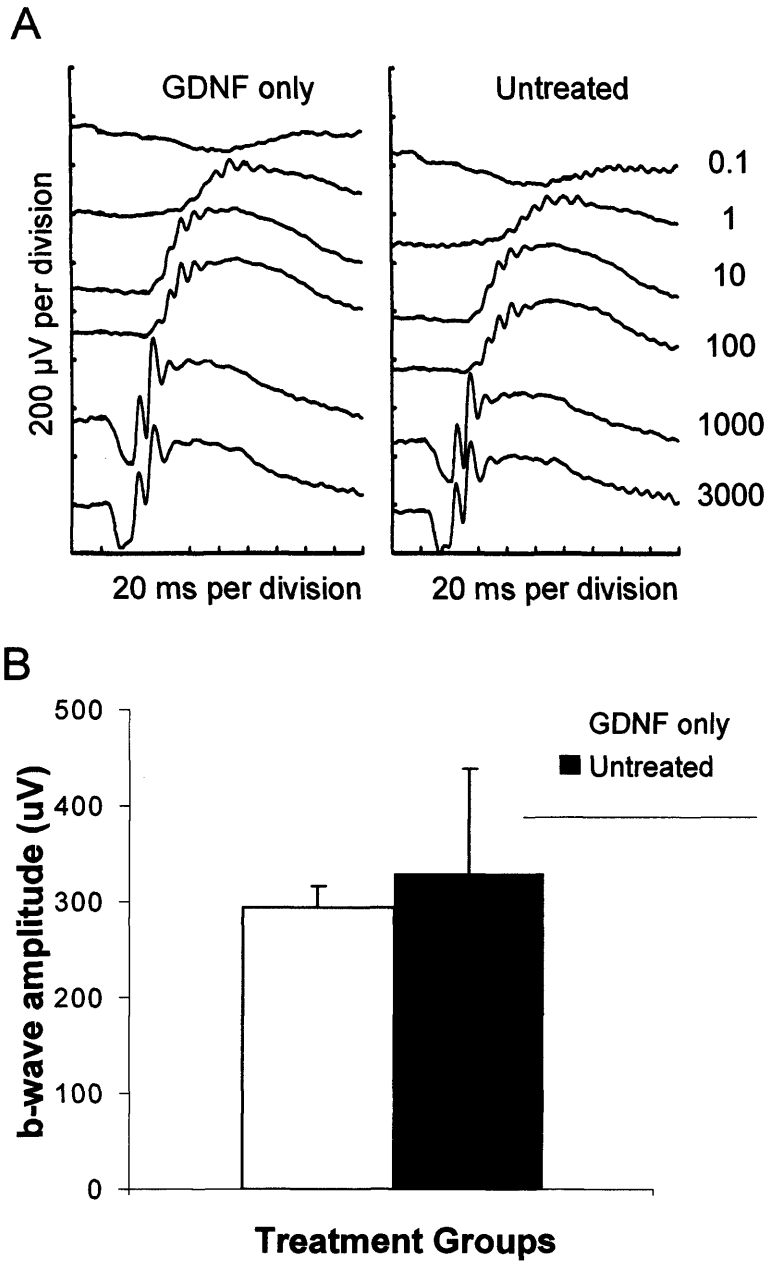


Fig. 6.7: AAV-mediated *GDNF* expression is well-tolerated in wild-type mice; Representative (A) and averaged (B) ERG recordings from six wild-type mice six weeks after sub-retinal injection of AAV.CBA.GDNF show normal waveform and a- and b-wave amplitudes in injected eyes (A left panel) when compared with uninjected contralateral eyes (A right panel). (B) No significant difference is seen between averaged b-wave values from the animals described in (A) ($p > 0.05$).

We therefore treated both *Prph2*$^{Rd2/Rd2}$ mice and RCS rats with the same vector. At post-natal day 10 one group of *Prph2*$^{Rd2/Rd2}$ mice received sub-retinal injections of AAV.CBA.GDNF alone in the right eye (1.5 µl), with the left eyes serving as uninjected controls. The GDNF-treated eyes showed no significant functional improvement as compared with the control eyes (**Figs. 6.8A and B**); this is to be expected, as in the absence of gene replacement therapy, *Prph2*$^{Rd2/Rd2}$ mice have negligible retinal function owing to the absence of photoreceptor cell outer segments. A further group of *Prph2*$^{Rd2/Rd2}$ mice received sub-retinal injections of a combination of AAV.CBA.GDNF and AAV.Rho.Prph2 viruses in the right eye, and of AAV.Rho.Prph2 virus alone in the left eye (n = 6). These mice were treated at post-natal day 10, and received a total of 1.5 µl of virus solution per hemisphere. At two and three months post injection, the eyes that received gene replacement in combination with GNDF show significantly enhanced b-wave amplitudes when compared with contralateral control eyes that received gene replacement alone; *Prph2*$^{Rd2/Rd2}$ eyes treated with combination of AAV.CBA.GDNF and AAV.Rho.Prph2 show 51 % higher b-wave amplitudes (p=0.007) than eyes injected with AAV.Rho.Prph2 only (**Fig. 6.8A and B**). When ERG recordings were taken four months post injection, despite a small b-wave being recorded from both eyes, there was no longer a significant difference between b-wave amplitudes from eyes treated with the combination of GDNF and gene replacement when compared with and eyes treated with gene replacement only (**Fig. 6.8C**).

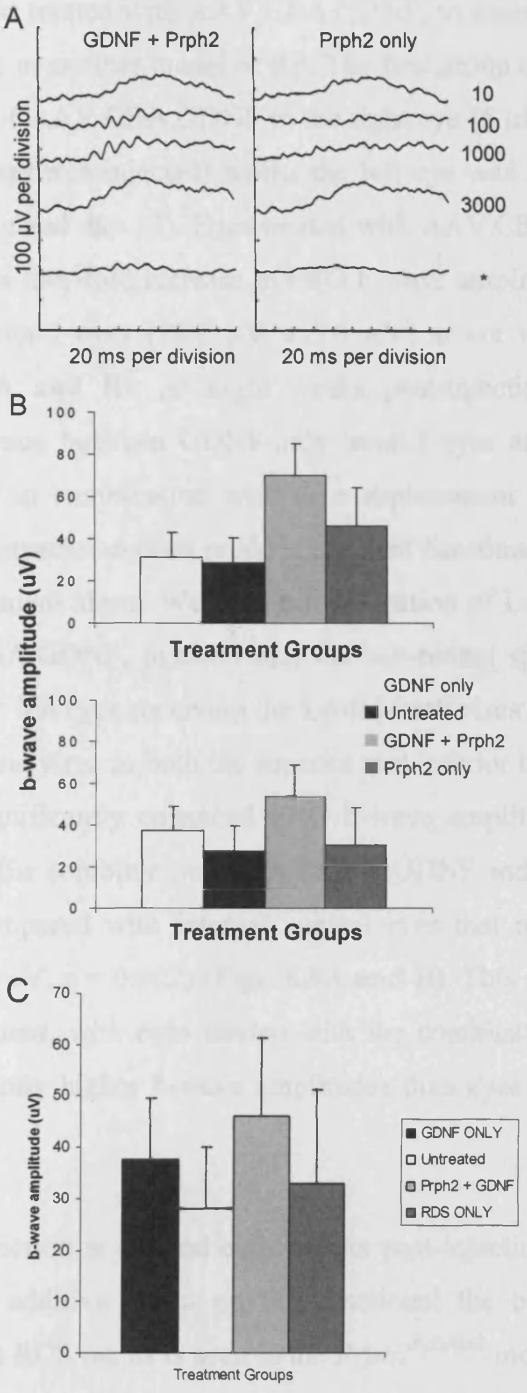


Fig. 6.8: AAV-mediated *GDNF* delivery enhances improvement in retinal function following gene replacement therapy in *Prph2*$^{Rd2/Rd2}$ mice; (A) Representative ERG traces recorded 2 months post-injection from *Prph2*$^{Rd2/Rd2}$ mice treated with combination of AAV.CBA.GDNF and AAV.Rho.Prph2 in one eye (left panel) and AAV.Rho.Prph2 only in the contralateral eye (right panel). For reference, traces at 100mcds/m^2 from a *Prph2*$^{Rd2/Rd2}$ mouse treated with GDNF only are shown (*). (B) Averaged b-wave values taken from the mice described in (A) (n = 6). Upper graph is from two months post-injection, lower graph is three months post-injection. (C) Four months post-injection, there is no longer a significant difference in b-wave amplitude between eyes treated with the combination of AAV.CBA.GDNF and AAV.Rho.Prph2 and those treated with AAV.Rho.Prph2 only ($p>0.06$).

RCS rats were also treated with AAV.CBA.GDNF, to assess the ability of GDNF to slow PR cell death in another model of RP. The first group of rats (n=6) received sub-retinal injections of AAV.CBA.GDNF in the right eye (5 µl per hemisphere, superior and inferior hemispheres injected) whilst the left eye was left uninjected (injections performed at post-natal day 12). Eyes treated with AAV.CBA.GDNF alone (66.8 µV ± 27.6 µV) show a four-fold increase in ERG b-wave amplitude when compared with untreated contralateral eyes (16.2 µV ± 5.9 µV) at six weeks post-injection ($p = 0.007$) (**Fig. 6.9A and B**). At eight weeks post-injection there is no longer a significant difference between GDNF-only treated eyes and untreated contralateral eyes. When used in combination with gene replacement therapy, however, AAV-mediated *GDNF* expression does produce a potent functional benefit when compared with gene replacement alone. We used a combination of Lenti.Mertk (50 ng p24 per ml) and AAV.CBA.GDNF, injected into the sub-retinal space of post-natal day 12 RCS rats, with the left eyes receiving the Lenti.Mertk virus alone. In all cases the rats received 5µl of total virus in both the superior and inferior hemispheres. At six weeks post-injection, significantly enhanced ERG b-wave amplitudes were recorded from eyes treated with the combination of AAV.CBA.GDNF and Lenti.Mertk (159.1 µV ± 36 µV) when compared with internal control eyes that received Lenti.Mertk only (105.9 µV ± 23.4 µV, $p = 0.002$) (**Figs. 6.9A and B**). This remained the case at eight weeks post treatment, with eyes treated with the combination of GNDF and Mertk showing significantly higher b-wave amplitudes than eyes treated with Mertk alone (**Fig. 6.9C**).

This enhanced function at six and eight weeks post-injection suggests that GDNF is able to exert an additive effect on the functional the benefit mediated by gene replacement in the RCS rat, as is seen in the *Prph2*$^{Rd2/Rd2}$ mouse. The effect of using a combination of neurotrophic gene delivery and gene replacement is marked in both models tested. In the *Prph2*$^{Rd2/Rd2}$ mouse, the combination of AAV-mediated *GDNF* expression and gene replacement therapy results in a 2.5-fold improvement in ERG b-wave amplitude when compared with no intervention, whereas treatment with the combination of AAV.CBA.GDNF and Lenti.Mertk results in almost an ten-fold increase in retinal function as compared with untreated eyes.

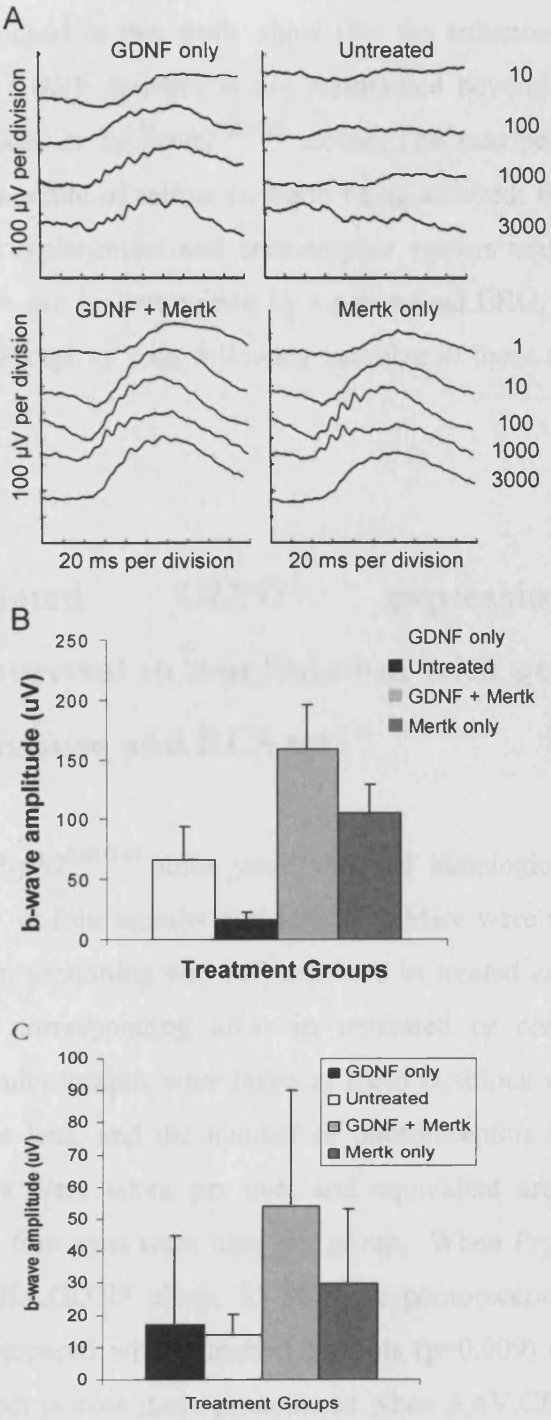


Fig. 6.9: AAV-mediated *GDNF* delivery enhances improvement in retinal function following gene replacement therapy in RCS rats; (A) Representative traces from RCS rats six weeks post-injection. Upper panel, traces from AAV.CBA.GDNF-only treated eye (left) and untreated control eye (right) from one rat. Lower panel, traces from an eye treated with a combination of AAV.CBA.GDNF and Lenti.Mertk (left), compared with traces from Lenti.Mertk-only treated eye (right). (B) Averaged b-wave amplitudes from animals described in (A). AAV-mediated *GDNF* delivery alone results in a four-fold increase in b-wave amplitude over untreated control eyes ($p=0.007$). When combined with Lenti-Mertk-mediated gene replacement, AAV-mediated *GDNF* expression results in a 50 % increase in b-wave amplitude ($p = 0.002$) as compared to gene replacement alone. (C) At 8 weeks post-injection, combination-treated eyes still show significantly higher b-wave amplitudes ($p = 0.03$) compared to Lenti.Mertk-treated eyes.

The later time points used in this study show that the enhanced function resulting from AAV-mediated *GDNF* delivery is not maintained beyond two months in the RCS rat and three months in the *Prph2*$^{Rd2/Rd2}$ mouse. This may be primarily due to the use of a pan-retinal measure of retinal function being assayed; the area of the retina treated with the gene replacement and neurotrophic vectors may well retain higher levels of function than can be determined by a pan-retinal ERG, and the histological preservation of photoreceptors seen following sacrifice at these time points suggests this could be the case.

6.6 AAV-mediated *GDNF* expression enhances photoreceptor survival in combination with gene replacement in *Prph2*$^{Rd2/Rd2}$ mouse and RCS rat

The treated *Prph2*$^{Rd2/Rd2}$ mice were assessed histologically following their final ERG recordings, at four months post-injection. Mice were sacrificed, their eyes processed for paraffin sectioning and cell numbers in treated areas were quantified and compared with corresponding areas in untreated or control eyes. For the quantification, photomicrographs were taken at fixed positions within injected areas using a 20x objective lens, and the number of photoreceptors in the field of view counted. Five images were taken per eye, and equivalent areas were used from internal control eyes; four eyes were used per group. When *Prph2*$^{Rd2/Rd2}$ mice were treated with AAV.CBA.GDNF alone, 25 % more photoreceptors were present in treated eyes when compared with untreated controls ($p=0.009$) (**Figs. 6.10A, 6.10B and 6.12A**). This effect is even more pronounced when AAV.CBA.GDNF is used in combination with gene replacement therapy in this model. Eyes treated with a combination of AAV.CBA.GDNF and AAV.Rho.Prph2 have on average 74 % more photoreceptors surviving at four months post-treatment than contralateral eyes that were treated with AAV.Rho.Prph2 only ($p=0.04$) (**Figs 6.1C, 6.10D and 6.12B**).

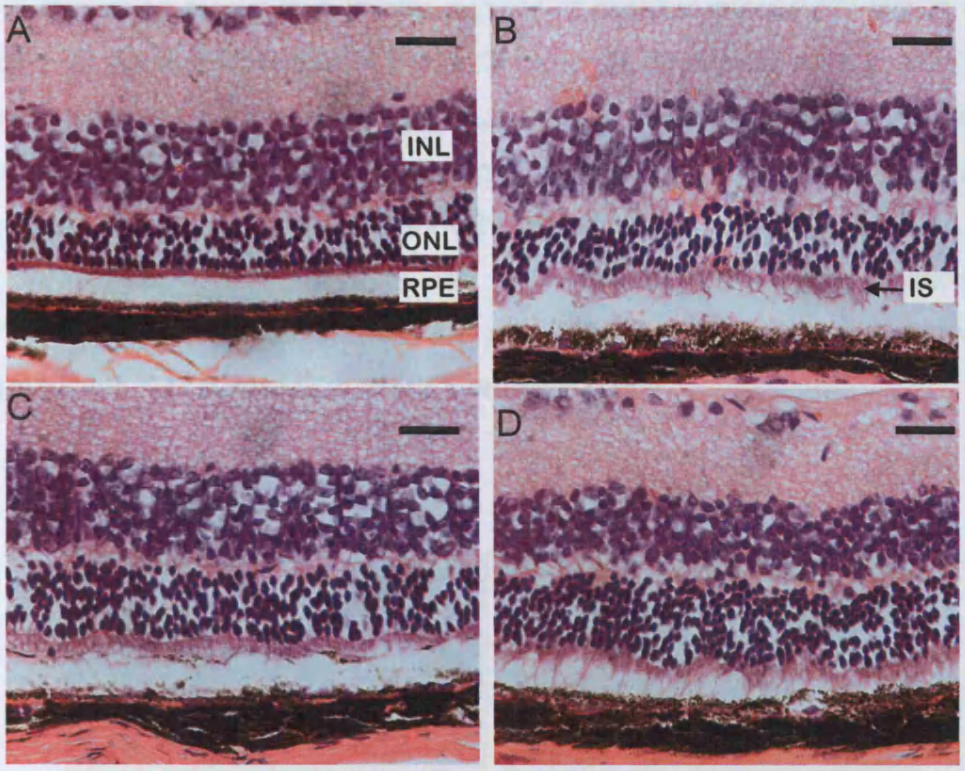


Fig. 6.10 – Enhanced preservation of photoreceptors in *Prph2*$^{Rd2/Rd2}$ mice following AAV-mediated *GDNF* delivery: Representative photomicrographs of H & E stained paraffin sections, taken using a 20X objective lens, from: (A) Untreated *Prph2*$^{Rd2/Rd2}$ eye; (B) AAV.CBA.GDNF-treated *Prph2*$^{Rd2/Rd2}$ eye; (C) AAV.Rho.Prph2 treated *Prph2*$^{Rd2/Rd2}$ eye; (D) AAV.CBA.GDNF and AAV.Rho.Prph2 combination-treated *Prph2*$^{Rd2/Rd2}$ eye. Greatest number of photoreceptors are present in the eyes treated with a combination of GDNF and gene replacement (D), more so than when eyes are left untreated (A), or when gene replacement alone is used in either model (C). INL, inner nuclear layer; RPE, retinal pigment epithelium; ONL, outer nuclear layer; IS, photoreceptor inner segments. Scale bar 25μm.

Again, comparing the combination of neuroprotection and gene replacement shows how potent this approach can be in this model, and supports the data from the assessment of retinal function; there is a four-fold increase in the number of photoreceptor cells surviving in eyes that received sub-retinal injection of the combination of AAV.CBA.GDNF and AAV.Rho.Prph2 when compared to untreated eyes. Importantly, the overall retinal morphology in eyes treated with GDNF, whether on by itself or in combination with gene replacement, was similar to that seen in wild-type mice.

RCS rat eyes were assessed three months post-injection to determine the histological effect of AAV-mediated *GDNF* expression in this model. Again, AAV-mediated

GDNF expression results in a greater number of photoreceptors surviving; in eyes treated with AAV.CBA.GDNF alone 64 % more photoreceptors were present than untreated contralateral eyes (**Figs. 6.11A, 6.11B and 6.12C**, p=0.006). Moreover, when used in combination with gene replacement therapy, AAV-mediated *GDNF* delivery allows a greatly enhanced survival of photoreceptor cells; **Figs. 6.11C, 6.11D and 6.12C** show that in RCS rat eyes treated with a combination of AAV.CBA.GDNF and Lenti.Mertk, 72 % more photoreceptor cells are present when compared with eyes treated with *MERTK* only (p=0.0004).

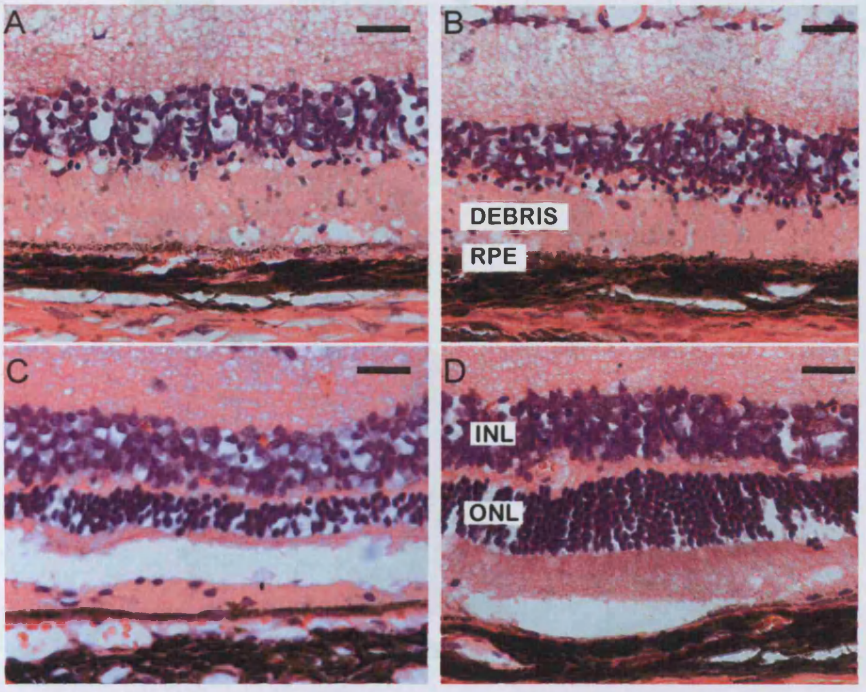


Fig. 6.11 – Enhanced preservation of photoreceptors in RCS rats following AAV-mediated *GDNF* delivery: Representative photomicrographs of H & E stained paraffin sections, taken using a 20X objective lens, from: (A) Untreated RCS eye; (B) AAV.CBA.GDNF-treated RCS eye; (C) Lenti.Mertk-treated RCS eye; (D) AAV.CBA.GDNF and Lenti.Mertk combination-treated RCS eye. Greatest number of photoreceptors are present in the eyes treated with a combination of GDNF and gene replacement (D), more so than when eyes are left untreated (A), or when gene replacement alone is used in either model (C). INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; DEBRIS, layer of photoreceptor debris that accumulates due to *Mertk* mutation in RCS rat. Scale bar 25μm.

There is a five-fold increase in the number of photoreceptors surviving when comparing eyes treated with the combination of AAV.CBA.GDNF and Lenti.Mertk to untreated eyes (**Fig. 5.12C**). This again emphasises the potent neuroprotective effect exerted by *GDNF*, in particular in combination with gene replacement therapy.

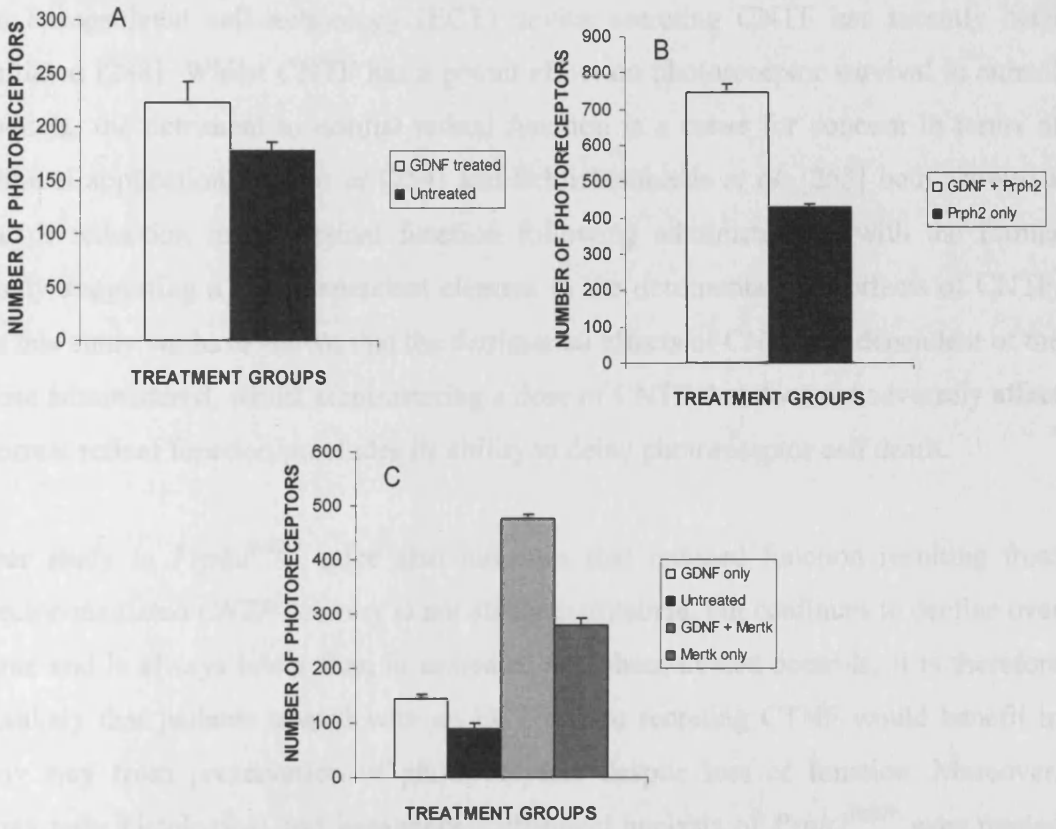


Fig. 6.12: Quantification of histological preservation of photoreceptors following AAV-mediated *GDNF* delivery; (A) In *Prph2*$^{Rd2/Rd2}$ mice, AAV-mediated *GDNF* delivery results in 25 % more photoreceptors surviving four months post-injection when compared with untreated internal control eyes (p=0.01). (B) Treatment with a combination of AAV.CBA.GDNF and AAV.Rho.Prph2 results in 74 % more photoreceptors surviving when compared with AAV.Rho.Prph2-mediated gene replacement therapy alone (p=0.04). (C) Sub-retinal injection of AAV.CBA.GDNF alone results in 64 % more photoreceptors surviving in the RCS rat when compared with uninjected eyes at three months post-injection (p=0.006), whilst injection of a combination of AAV.CBA.GDNF and Lenti.Mertk results in 72 % more cells surviving at the same timepoint when compared with contralateral eyes given Lenti.Mertk-mediated gene replacement therapy alone (p=0.001).

6.7 Discussion

CNTF is perhaps the most extensively studied neurotrophic molecule in the context of ocular therapeutics [125,252-255,285-287]. Transplantation into the vitreous of encapsulated cells stably transfected with the *CNTF* gene [287] or intraocular injection of viral vectors expressing *CNTF* have prolonged photoreceptor cell survival by up to three months [253-255]. Indeed, a small phase I clinical trial of an encapsulated cell-technology (ECT) device secreting CNTF has recently been initiated [288]. Whilst CNTF has a potent effect on photoreceptor survival in animal models, the detriment to normal retinal function is a cause for concern in terms of clinical application; Bok *et al* [254] and Schlichtenbrede *et al.* [255] both showed a major reduction in pan-retinal function following administration, with the former study suggesting a dose-dependent element to the detrimental side effects of CNTF. In this study we have shown that the detrimental effects of CNTF are dependent of the dose administered, whilst administering a dose of CNTF that does not adversely affect normal retinal function precludes its ability to delay photoreceptor cell death.

Our study in *Prph2*$^{Rd2/+}$ mice also indicates that reduced function resulting from vector-mediated *CNTF* delivery is not stably maintained, but continues to decline over time and is always lower than in untreated and sham-treated controls. It is therefore unlikely that patients treated with an ECT device secreting CTNF would benefit in any way from preservation of photoreceptors despite loss of function. Moreover, long-term histological and immunohistochemical analysis of *Prph2*$^{Rd2/+}$ eyes treated with CNTF demonstrated damage to photoreceptors; specifically, the shutoff of cone opsin expression is of concern in terms of clinical application, as patients receiving CNTF-mediated treatment depend on a central island of cone-rich macula for residual vision.

Further work is required to investigate the causes of this long-term deleterious effect on photoreceptors. We need to determine whether long-term *CNTF* expression actively induces photoreceptor apoptosis, or whether another mechanism is responsible for fewer photoreceptors surviving in *Prph2*$^{Rd2/+}$ eyes treated with CNTF than in untreated eyes. The mechanism behind cone opsin shutoff is also unclear;

previous studies using AAV-mediated *CNTF* expression have reported morphological changes in inner retina nuclei, suggestive of a change in the gene expression in these cells. In all, these data suggest that the deleterious effects of CNTF outweigh any potential beneficial effects, and urge caution against the use of CNTF-mediated treatment for retinal degeneration in patients.

In the case of GDNF, a strong neuroprotective effect has also been demonstrated in rodent and primate models of Parkinson's disease [289]. However, Phase II clinical trials in which Parkinsonian patients received intracranial infusion of recombinant GDNF protein were halted as no clinical benefit was observed. Evidence from another study suggested that long-term GDNF infusion was well tolerated and efficacious in ameliorating symptoms [290], suggesting that the decision to discontinue the larger-scale trial may have been premature. Viral vector-mediated delivery of *GDNF* has been used to delay Parkinsonian symptoms in rodents and non-human primates, although *GDNF* gene delivery appears to cause overexpression-related side effects in both Parkinsonian and normal brain tissues [291]; components of the dopamine synthesis pathway in striatal neurones are directly downregulated by GDNF, leading to reduced dopamine production. This has lead to the development of inducible viral vectors to deliver the neurotrophic factor [292] the use of which is designed to prevent the effects of continuous *GDNF* expression.

Previous studies that have examined the effects of prolonged *CNTF* transgene expression in the eye have reported changes in the morphology of photoreceptor cell nuclei and have suggested that effects on the outer retina or re-modelling of the inner retina might be responsible for the reduction in retinal function [254,255]. We did not notice any obvious changes in the inner or outer retinal appearance following injection of vector expressing *GDNF*. This suggests that AAV-mediated *GDNF* expression is better tolerated than AAV-mediated *CNTF* expression, and supports evidence from previous work that the mechanisms of action of GDNF are distinct from those of CNTF [293]. Evidence from our study further demonstrates that whilst exerting a neuroprotective effect on degenerating photoreceptors, CNTF causes loss of retinal function, whereas GDNF is able to significantly slow photoreceptor loss without the concomitant detrimental side effects. These data represent the first evaluation of a combination approach to viral vector-mediated retinal gene therapy,

whereby a neurotrophic factor is shown to act synergistically with gene replacement to provide enhanced cell survival over gene replacement or growth factor alone. Due to its apparent lack of deleterious side effects GDNF may in fact be a more appropriate candidate to be carried through into large animal models and eventually patient trials, although further work dissecting the precise mechanisms underlying the neuroprotective effect exerted by GDNF is needed before such a clinical application can be considered.

7. Discussion

7.1 Improving gene replacement therapy in rodent models of retinal degeneration

The experiments discussed in **Chapters 3 and 4** were aimed at developing improved gene replacement strategies in five clinically applicable models of retinitis pigmentosa: the RCS rat, the *rd12* mouse, the *Prph2*$^{Rd2/Rd2}$ mouse, the *rd1* mouse and the *Rho*$^{-/-}$ mouse. One of the major objectives, that of demonstrating efficient transgene expression using novel integrase-deficient viral vectors in rodents, was achieved. We were also able to show efficacy of these integrase-deficient vectors in mediating gene replacement therapy in the RCS rat and *rd12* mouse. In the first instance, these data are to our knowledge the first example of effective, stable gene replacement therapy mediated by integrase-deficient vectors. The use of lentiviral vectors carrying pleiotropic *integrase* mutations has several advantages over both integrating lentiviruses and over other vectors whose genomes remain episomal. Because these novel lentiviral vectors mediate gene transfer to post-mitotic cells in the absence of integration into host chromosomal DNA, they represent a significant advance in the biosafety of lentiviral vectors; the risk of insertional mutagenesis is greatly reduced compared to integrating retroviral and/or lentiviral vectors, such that these novel vectors have much less oncogenic potential. Unfortunately the gene replacement therapy in which insertional oncogenesis was shown to cause significant adverse effects (*IL2RG* gene replacement in patients with X-SCID, see **Chapter 1**) would presumably not be a suitable application for integrase-deficient lentiviral vectors, as the episomal vector DNA would be lost from the rapidly dividing haematopoietic stem cells transduced in this therapeutic regimen. Nonetheless, there is great potential for the use of integrase-deficient vectors in the treatment of diseases affecting post-mitotic tissues such as muscle, the CNS and the eye, whereby the lack of integration does not reduce the high efficiency and stability of gene expression that make lentiviral vectors very efficient gene therapy agents. It is this combination of increased biosafety and preservation of therapeutic efficacy that is most promising in terms of applying lentiviral gene therapy to humans.

We showed that by using an AAV2 vector pseudotyped with the AAV5 capsid, we were able to enhance the functional improvement following gene replacement therapy in the *Prph2*$^{Rd2/Rd2}$ mouse (**Chapter 5**); based on studies comparing the kinetics of transgene expression between different AAV serotypes [265], this was likely due to a higher proportion of cells being transduced, and the onset of transgene expression from AAV2/5.Rho.Prph2 being faster than that from the AAV2/2.Rho.Prph2 used in previous studies. Pseudotyped AAV vectors have been previously shown to mediate rapid, stable reporter gene expression in the eye following sub-retinal delivery in wild-type mice [264,265], and in the muscle using intra-articular delivery in a model of arthritis [294]. Therapeutic gene delivery using AAV2/5 has also been investigated; *GALC* gene replacement therapy in twitcher mice, which are a model of inherited globoid-cell leukodystrophy, results in higher GALC protein production, greater preservation of CNS morphology and greater longevity of mice when mediated by AAV2/5 when compared with AAV2/2 [294]. Also, AAV2/5 was shown to mediate effective gene replacement in *RPE65*$^{-/-}$ dogs [295], a model of LCA caused by RPE65 deficiency. Thus, the therapeutic potential of AAV2/5 has been established, and the data presented here comparing AAV2/5.Rho.Prph2 with AAV2.Rho.Prph2 (**Chapter 5**) confirm that gene replacement therapy using AAV2/5 results in significantly improved function when compared with AAV2/2. Notwithstanding this finding, we report that there does not appear to be significantly more photoreceptors surviving in AAV2/5-treated eyes when compared with AAV2/2-treated eyes. A study in which AAV2/5 and AAV2/2 were administered to murine CNS would seem to support our data. The authors of this study conclude that although more efficient transduction was achieved with AAV2/5 encoding a reporter gene when compared to AAV2/2, protection from cell death following axotomy was no greater following therapeutic gene delivery of nerve growth factor (NGF) by AAV2/5 than by AAV2/2 [296]; this was attributed to having reached a ceiling of NGF protein levels with the AAV2/2 vector, meaning that the more efficient pseudotyped vector is unable to mediate enhanced production of NGF, which prevents AAV2/5-treated neurones from surviving longer. It would be of considerable interest to see whether the expression of *Peripherin* had reached a similar peak using AAV2/2, or whether treatment with AAV2/5 does lead to increased *Prph2* transcipt production. Despite having shown that there is a significant improvement in the ERG b-wave obtained from AAV2/5.Rho.Prph2-treated eyes compared with AAV2/2-treated eyes, our study

suggests that there is not a reduced loss of photoreceptors in eyes treated with AAV2/5. It would appear that this is because gene replacement therapy does not stop photoreceptors from dying by apoptosis in this model of retinal degeneration. Nonetheless, further investigation of the effect of AAV2/5-mediated gene replacement therapy in this model is warranted. We sacrificed the treated animals five months after injection, at a time when the AAV2/5-treated eyes did not have significantly better ERG b-wave amplitudes than AAV2/2-treated eyes. It is conceivable that we may have seen more photoreceptors surviving in AAV2/5-treated eyes than in AAV2/2-treated eyes had we examined a cohort of eyes at 10 weeks post-injection, when the former had on average 27% greater b-wave amplitudes than the latter. This will be investigated as part of the studies that follow the work presented here. In addition, we were unable to fully test the effect of gene replacement therapy on cone function as part of the studies presented here. The ERG equipment used to monitor retinal function in our treated mice has now been improved in order to investigate cone function, and in addition the use of novel LED-based ERG techniques enables reliable measures of cone function to be taken. We have demonstrated that the novel fragment of the mouse opsin promoter (MOPS) described in **Chapter 5** drives transgene expression in rods, but not in cones. As part of the further work to be carried out following the studies presented here, alternative promoter sequences that mediate expression in both rods and cones need to be investigated, with the aim of administering gene replacement to models such as the *Prph2*$^{Rd2/Rd2}$ mouse and assaying their cone function. As many RP patients in end-stages of disease depend on their cone-mediated vision once the peripheral retina has atrophied, it is of great importance from a clinical perspective to develop gene replacement therapies that specifically enhances cone function.

Whilst were able to demonstrate that AAV2/5 mediates enhanced functional improvement in the *Prph2*$^{Rd2/Rd2}$ mouse, the other models of retinal degeneration we studied appear to benefit less from AAV2/5-mediated gene replacement therapy. The failure of AAV2.5.CMV.βPDE to mediate efficient transgene expression *in vivo* in the *rd1* mouse, despite the expression cassette being effective *in vitro*, shows that certain models of retinal degeneration may be less amenable to gene replacement than others. We cannot rule out the possibility that our particular rAAV preparation was either rendered non-infectious or contaminated with toxic by-products during

preparation, although we did repeat the virus isolation and obtained the same result. Previous attempts at treating the *rd1* mouse using gene replacement therapy have also proved largely unsuccessful, either through use of adenoviral vectors [178,227] which have subsequently been shown not to transduce photoreceptors [168], through use of lentiviral vectors [228] for which the same applies [199] or through low-level protein production following administration of relatively low-titre AAV ($1x10^7$ infectious particles per ml, [230]). It appears, therefore, that achieving rescue in the *rd1* mouse is challenging, and that both more comprehensive reporter gene studies and further advances in vector production may be necessary to rescue this model of retinal degeneration.

7.2 Neuroprotection in combination with gene replacement therapy may be an effective treatment strategy for retinitis pigmentosa

Preventing photoreceptor cell death is vital to the success of any treatment for retinal degeneration; correcting a molecular defect, even if it results in ameliorating the function of the tissue being treated, will only produce a transient treatment effect if photoreceptors continue to die at the same rate as before or without treatment. This has been effectively demonstrated with gene replacement studies in the $Prph2^{Rd2/Rd2}$ mouse, whereby an initial improvement in retinal function begins to decline nine weeks post-treatment [246], caused by the failure to slow photoreceptor loss [244]. Hence, a large number of studies have investigated a different approach to gene therapy for retinitis pigmentosa, neuroprotection – the delaying or preventing of photoreceptor apoptosis by the use of neurotrophic factors. Many of the first studies using this approach were concerned only with allowing photoreceptors to survive for longer following administration of a neurotrophic factor, either as recombinant protein [124,125] or by vector-mediated delivery of the gene encoding the neurotrophic factor [247,252]. These studies demonstrated that, amongst others, ciliary neurotrophic factor can mediate some protection from cell death in models of retinal degeneration, but that this protection is short-term. Longer-term protection from cell death has been demonstrated in rodents that carry mutant human transgenes

for *Rhodopsin* [253] and *Peripherin* [254], but these studies also demonstrated that CNTF has detrimental effects on retinal electrophysiology that cased major concern in terms of translating this type of therapy to a clinical setting. One crucial aspect of these studies is that no correction of the underlying molecular defect was attempted alongside the administration of the neurotrophic agent; the aim of these studies was simply to prolong photoreceptor cell survival, but a treatment in which cells survive longer but still have little or no function is unlikely to be of benefit to patients. Attempts were therefore made to assess the effect of combining neuroprotection and gene replacement therapy. In this study Schlichtenbrede *et al.* showed that CNTF administration resulted in reduced retinal function both in normal animals and in $Prph2^{Rd2/Rd2}$ mice that had received gene replacement therapy. The experiments described in **Chapter 6** follow on from these previous studies by demonstrating that CNTF causes dose-dependent detrimental side effects to retinal function, and that reducing the dose of CNTF abrogates its neuroprotective function. Moreover, we report long-term detrimental effects on cone opsin expression, electrophysiology and retinal morphology following long-term exposure to CNTF in $Prph2^{Rd2/+}$ mice. It remains to be seen whether these finding will be replicated in the patients that have received CNTF as part of a clinical trial, although our findings do not support this approach as a clinical therapy.

We have shown, nonetheless, that neuroprotection in combination with gene replacement therapy is feasible, and that slowing of photoreceptor cell death can be combined with restoration of an underlying molecular defect without detrimental side effects. Our results using AAV-mediated *GDNF* expression indicate that GDNF may be a more suitable candidate than CNTF for further investigation as a neuroprotective agent; we have shown that expression of *GDNF* does not reduce normal retinal function, and enhances gene replacement as shown by significantly higher ERG b-wave amplitudes and significantly more photoreceptors surviving at the latest time points assessed. Nevertheless, many more issues need to be addressed before the use of GDNF in clinical trials can be considered. The improvement in retinal function appears to remain transient, despite there being more photoreceptors present in GDNF-treated eyes than in untreated eyes or those that received gene replacement alone. There are a number of alternative explanations for this finding. It may be that by using a pan-retinal measure of retinal function, we may be unable to detect an area

of GDNF-treated retina that has preserved function for longer than in other areas in the same eye. Consider that some patients with a completely extinguished pan-retinal scotopic ERG retain photoreceptor function in a small sector of retina, meaning their visual acuity is preserved in a narrow visual field to the extent that their quality of life is maintained [297]. It is conceivable that a therapy such as AAV-mediated *GDNF* expression, in combination with an efficient gene replacement therapy, could achieve preservation of structure and function in given areas of the retina (of particular interest would be the preservation of photoreceptors in the macula), and this could be sufficient to constitute an effective clinical therapy. The use of visual acuity tests, multifocal ERG and microperimetry would aid the assessment of whether this is the case in patients. To do the same in rodents is more challenging, as most measures of visual function used in rats and mice average measurements from across the retina. One technique that may allow function in discrete areas of the retina to be measured and compared would be the use of a multi-electrode array (MEA) on retinal explants to record the response from the ganglion cells or inner retina following stimulation with light [298].

As the studies presented here demonstrate, the success or otherwise of gene replacement therapy and neuroprotection in animal models of retinal degeneration depend upon several factors. The development of novel vector systems that mediate increasingly efficient and safe transgene delivery is of great importance, but is not the only factor involved. Certain models of disease appear to be more amenable to viral gene therapy than others, and it may be that the nature of the causative mutation underlies this. Some models of RP have shown long-term protection from cell death following gene replacement therapy alone; disease correction in models of RP caused by mutations in *Rpe65, RPGRIP* and *Rs1* results in retardation of apoptotic cell death. Models of RP caused by more severe biochemical insults to photoreceptors and/or the RPE are inherently difficult to treat, because it appears that photoreceptors commit to apoptotic cell death if they have severe disturbances in their metabolic capabilities or structure, whereas those models that have more subtle insults to photoreceptor metabolism appear easier to treat. This is where neuroprotection may have most impact; we have seen that gene replacement alone is not capable of slowing cell death in the $Prph2^{Rd2/Rd2}$ mouse, and is not effective at doing so to any great extent in the RCS rat. Our studies show that by using a combination of gene replacement and

neuroprotection, long-term preservation of photoreceptors can be achieved. Thus a similar combination may be useful in other models of disease in which gene replacement therapy does not appear sufficient to mediate long-term rescue.

Appendix I

PCR primer sequences, MOPS promoter:

Outer pair: 5' TTTAGGGCCCAAGGGTTCCAGTCG 3', TM = 64.7 °C

5' CGTTCATGGCTGCGGCTCTCG 3', TM = 64.5 °C

Inner pair: 5' CAGACTGACATGGGGAGGAAT 3', TM = 57.2 °C

5' CTCCCACAGCGCAACTCC 3', TM = 53.7 °C

Immunohistochemistry conditions:

Chicken anti-mouse cone opsin (mixture of anti-S-opsin and anti-L/M-opsin antibodies, **Fig. 5.7** and **Fig. 6.5**): 1/5000 in TBS-T. Secondary antibody: Alexa 546-conjugated goat anti-chicken IgG (Molecular Probes, UK), 1/200 in TBS-T.

Biotinylated peanut agglutinin (PNA, **Fig. 5.7** and **Fig. 6.5**): 1/200 in TBS-T. Streptavidin-conjugated Alexa 488, 1/250 in TBS-T

Mouse monoclonal anti-Rhodopsin (**Fig. 5.16**): 1/100 in TBS-T. Secondary antibody: Alexa 546-conjugated goat anti-mouse IgG 1/500 in TBS-T

Rabbit anti-mouse β-PDE serum (**Fig. 5.14**): 1/500 in TBS-T. Secondary antibody: Alexa 546-conjugated goat anti-rabbit IgG 1/150 in TBS-T.

Rabbit polyclonal anti-mouse βPDE antibody (**5.15**): 1/500 in TBS-T. Secondary as above.

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